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S100 Proteins

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Synonyms

Allergy; Biomarkers; Calcium-binding; Cancer; Cardiomyopathy; Clinical chemistry; Diagnostics; Drug targets; EF-hand; Head injury; Inflammation; Laboratory medicine; Zinc-binding

Definition

S100 proteins are small acidic proteins and are characterized by two distinct EF-hand calcium-binding motifs with different affinities. They also bind zinc, copper, and iron at positions distinct from the Ca^{2+}-binding sites resulting in modulation of their activities. Most S100 genes are organized in a gene cluster on human chromosome 1q21, the basis of the S100 nomenclature approved by the HUGO nomenclature committee (Marenholz et al. 2004). Today, members of the S100 protein family are routinely used in Practical Laboratory Medicine to analyze and monitor disease conditions and are important diagnostic and prognostic biomarkers (Heizmann 2019a, b).

Basic Characteristics

S100 proteins constitute the largest subgroup within the EF-hand protein family. They are involved in many cellular activities (Marenholz et al. 2004; Heizmann 2019a, b; Leclerc and Heizmann 2011; Donato et al. 2002) and are mostly in the form of homo- or hetero-dimers but can also form tetramers and hexamers (Moroz et al. 2002; Ostendorp et al. 2007; Spratt et al. 2019). The crystal structures of S100Z and of other S100 proteins have recently been reviewed (Calderone et al. 2019).

S100A3 is a unique member of this protein family. It binds Ca^{2+} with poor but Zn^{2+} with an exceptionally high affinity. The crystal structure of S100A3 is shown in Fig. 1 and described in detail (Fritz et al. 2002). Molecular genetic investigations, performed on patients with pulmonary fibrosis (one of the leading indications for lung transplantation), revealed a hypomorphic variant in *S100A3* and a novel truncated mutation in *S100A13,* both segregating with the disease in an autosomal manner (Al-Mutairy et al. 2019). These were the first mutations detected in any of the S100 protein family members and suggest a calcium- or zinc-dependent therapeutic approach for the management of this disease of unknown etiology.

Besides their distinct intracellular functions, certain S100 proteins are secreted from cells and exert cytokine-like functions through the binding

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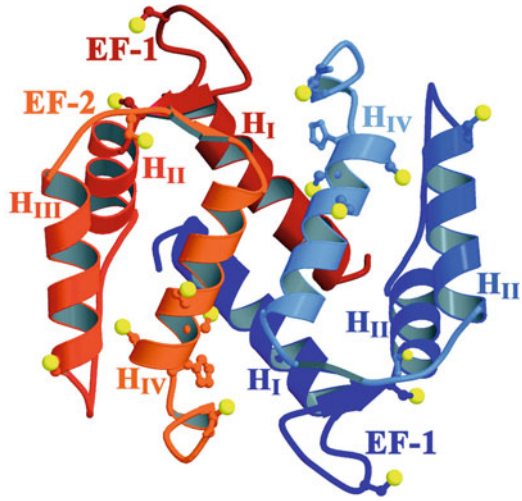

S100 Proteins, Fig. 1 Overall structure of the Ca^{2+}-free S100A3 dimer. The subunits are shown in *red* and *blue,* respectively. The N termini are shown in dark colors and the C termini in bright colors. The Cys and His residues are shown as a *ball* and *stick* model. This research was originally published in the *Journal of Biological Chemistry*, G. Fritz et al. (2002) The Crystal Structure of Metal-free Human EF-Hand Protein S100A3 at 1.7-Å Resolution 277, 33092-33098 ©the American Society for Biochemistry and Molecular Biology

and activation of cell surface receptors such as the Receptor for Advanced Glycation End Products (RAGE), Toll-like Receptor 4 (TLR-4), the ErbB4 receptor, the dopamine D2 receptor, amyloid-β, and annexins (Marenholz et al. 2004; Heizmann 2019a, b; Leclerc and Heizmann 2011; Donato et al. 2002; Moroz et al. 2002; Ostendorp et al. 2007; Spratt et al. 2019).

Clinical Importance of S100 Proteins in Human Diseases

Cardiology

S100A1 is predominantly expressed in the heart and a decreased cardiac expression of S00A1 is characteristic of heart failure. S100A1 gene transfer to restore S100A1 protein levels in the failing myocardium is suggested as a future therapeutic strategy to support the injured heart and to reverse or prevent heart failure (Pleger et al. 2007).

S100A2 has the potential to differentially influence Ca^{2+}-cycling and contractility of cardiomyocytes. Therefore, S100A2 and S100A4, in addition to S100A1, may be further candidates for a differentiated heart failure therapy promoting survival of cardiac myocytes (Wang et al. 2014).

Oncology

S100 proteins associated with cancer progression, diagnosis, and treatment are listed in Table 1.

S100B is the best-studied melanoma biomarker incorporated into the American Joint Committee on Cancer (AJCC) melanoma staging system. S100B is routinely analyzed in practical laboratory medicine and elevated S100B levels closely correlate with a poor survival rate.

S100A2-A6. A prognostic significance of S100A2 in laryngeal squamous-cell carcinoma allows discrimination of high-and low-risk patients in the lymph node-negative subgroup for a better adjusted therapy. S100A2 has been found to be a marker for early stages of lung carcinogenesis.

S100A4 (metastasin), a key player in tumor progression and metastasis, is by far the best studied and prominent member of the S100 protein family. The importance of S100A4 in cellular invasion and metastasis is exemplified by its presence in a great variety of different types of cancer, as listed in Table 1. S100A4 can also act via an extracellular route, as an angiogenic factor inducing tumor progression. Inhibiting the process of tumor angiogenesis is a possibility by blocking either S100A4 secretion or its extracellular binding to targets/receptors.

S100A7 (psoriasin) and the homologous protein *S100A15* (koebnerisin) in psoriatric keratinocytes are associated with several malignant tumors.

S100A8/A9 proteins (calprotectin/Mrp8 and Mrp 14) are involved in different types of cancer. Their expression and proposed cytokine-like functions indicate key roles in inflammation-associated cancer and represent promising biomarkers to evaluate the risk potential of various types of cancer in molecular pathology.

S100A11 may become important for the diagnosis and treatment of ovarian cancer.

S100A13 was identified in thyroid tumors and its growth and invasion. A proteomics analysis of cutaneous malignant melanoma (CMM) revealed an association between the expression of

S100 Proteins, Table 1 S100 proteins in oncology

Proteins	Cancer association
S100B	Melanoma metastasis
S100A1	Ovarian cancer
S100A2	Lung carcinogenesis, laryngeal squamous-cell carcinoma
S100A2-A6	Classification of brain tumors
S100A3	Hepatocellular carcinoma, gastric cancer
S100A4/metastasin	Colon, rectal, gastric, ovarian, breast cancers; melanoma; oral squamous cell carcinomas
S100A7/psoriasin	Ovarian, cervical, pancreatic cancers
S100A8/A9/calprotectin	Inflammation-associated cancer, breast, colorectal cancers; ALL[a]; CLL[b]; AML[c]; cutaneous squamous, laryngeal, hepatocellular carcinoma, head, neck, bladder cancer
S100A11	Breast, ovarian, pancreatic cancers
S100A13	Thyroid tumors, cutaneous malignant melanoma
S100A14	Cervical, breast, gastric cancers
S100A16	Colorectal, breast cancers
S100 P	Breast cancer, pancreatic ductal adenocarcinoma, nasopharyngeal carcinoma

[a]Acute lymphoblastic leukemia
[b]Chronic lymphocytic leukemia
[c]Acute myeloid leukemia

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S100A13 and chemotherapy resistance. CMM is commonly unresponsive to standard dacarbazine (DTIC) and temozolomide (TMZ) chemotherapy, and the use of S100A13 as a predictive marker of therapy response would be of great clinical help.

S100A14 is overexpressed in several cancer tissues regulating proliferation, migration, and invasion and suppressing metastasis (e.g., in gastric cancer).

S100P is expressed in many pancreatic tumors, associated with growth and aggressiveness of this cancer type.

Inflammation, Autoimmune Diseases and Allergies

S100B is correlated with active vitiligo depigmentation (Birlea 2017), the most common depigmenting disorder. Serum levels of S100B were increased in patients with active non-segmental vitiligo and associated with disease progression. These findings imply that S100B may be a biomarker for this disease and a new target for treatment.

S100A4/metastasin is implicated in metastatic tumor progression and chronic inflammation. S100A4 induced the expression of acute-phase responsive proteins, serum amyloid A (SAA1), and SAA3 proteins and cytokines in an organ-specific manner. SAA proteins in connection with S100A4 serve as a link between inflammation and tumor progression (Hansen et al. 2014; Ambartsumian et al. 2019). S100A4 was also identified as a candidate gene in allergy. Treatment with an anti-S100A4 antibody resulted in decreased signs of allergy in a mouse model and in allergen-challenged T cells from allergic patients. This strategy may be generally applicable to identify diagnostic and therapeutic candidate genes in allergy.

S100A7/psoriasin, localized in epithelial cells, regulates cell proliferation and differentiation. An increase of S100A7 expression is found in response to inflammatory stimuli, such as in psoriasis (a chronic inflammatory autoimmune-mediated skin disease). This suggests that S100A7 may be a therapeutic target in psoriasis (D'Amico et al. 2016). Serum levels of S100A7 and S100A15 are potential markers of artherosclerosis in patients with psoriasis (Awad et al. 2018).

S100A8 and S100A9 proteins are characterized by a unique expression pattern with strong prevalence in cells of myeloid origin and are associated with a number of chronic inflammatory diseases (Holzinger et al. 2019). Sensitive and automated ELISA calprotectin (S100A8/A9) assays are routinely used in Clinical Chemistry Laboratories to diagnose the two main forms of inflammatory bowel diseases, Crohn's disease and Ulcerative colitis.

S100A11 (calgizzarin) was associated with rheumatoid- and osteoarthritis and is an additional biomarker to S100A/A9 and S100A12 for a more tailored diagnosis of inflammatory diseases.

S100A12/calgranulin C is associated with the Kawasaki disease and Mooren's ulcer as well as atherosclerosis.

Brain Injury and Neuropathologies

S100 proteins were first described as small acidic proteins in a 100% saturated ammonium solution of a brain extract. This extract mainly contained the S100A1 and S100B proteins. S100B represent 0.2% of total brain proteins, mainly synthesized by astrocytes, oligodendrocytes, and Schwann cells. S100 protein members (mainly S100B) are associated with neurodegenerative diseases such as Alzheimer´s disease, Down syndrome and in some psychiatric disorders such as schizophrenia (Table 2) and are routinely used in Practical Laboratory Medicine as neurobiomarkers for head injuries of children and adults.

Drugs

Developing inhibitors against S100B as an antagonist (Khan et al. 2018; Syed et al. 2018) to block the interaction with RAGE is a promising goal for future pharmaceutical interventions and cancer treatment. Targeting the S100A7/A15-receptor axis is suggested as a new targeted strategy for preventing the invasion and metastasis of these types of cancer. Another therapeutic strategy is using neutralizing monoclonal antibodies against S100A7 for cancer treatment. As a consequence

S100 Proteins, Table 2 S100 proteins in brain disorders

Proteins	Brain pathologies
S100B	Neurobiomarker for head injury in high-risk fetuses, newborns, children, and adults biomarker in asphyxiated infants treated with hypothermia intracerebral hemorrhage and ischemic stroke Alzheimer's disease, multiple sclerosis, psychiatric disorders (schizophrenia) acute cerebrovascular insults, seizures, delirium glyphosate and glufosinate poisoning pesticide-induced neurotoxicity
S100A4	Axonal regeneration, neuronal plasticity
S100A6	Amyotrophic lateral sclerosis
S100A7/ A15	Antimicrobial proteins in the CNS
S100A10 (p11)	Biomarker for monitoring the severity of Parkinson's disease

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of the various proinflammatory properties of S100A8/A9 proteins, strategies targeting these molecules are an option for anti-inflammatory therapies, by in vivo administration of S100 antibodies, or by therapeutic drug targeting to inhibit the release of these cytokine-like molecules at sites of inflammation (Vogl et al. 2018).

The antiallergic drug cromolyn binds to S100P preventing RAGE activation inhibiting pancreatic tumor growth and metastasis in animal models. Cytotoxic treatment using cromolyn in combination with gemcitabine or applying a small RAGE antagonistic peptide appears to be a promising to treat pancreatic and possibly other types of cancer. Covalent small molecule inhibitors of S100B have been developed that inhibit S10B-p53 complex and restore active p53 in malignant melanoma (Cavalier et al. 2014). A similar approach was to target the estrogen receptor/S100 interface by a short peptide derived from S100P in order to reduce regression of tumor growth receptor positive breast cancer (Lee et al. 2016; Cho et al. 2016).

Outlook

Today, members of the S100 protein family are routinely used in Practical Laboratory Medicine to analyze and monitor disease conditions and are important diagnostic and prognostic biomarkers. S100 proteins and their targets/receptors are also considered as drug targets for future treatments. So far, only a small number of S100 proteins have been explored for their clinical relevance although more and more human diseases (Tables 1 and 2),

as well as, for example, obesity (Riuzzi et al. 2019) and the sexually-transmitted infection gonorrhea (Maurakis et al. 2019) are linked to S100 proteins. Methods of choice for a simultaneous analysis of Ca^{2+}-binding proteins and their post-translational modifications are the mass spectrometry and the focal molography (a next-generation biosensor that visualizes specific biomolecular interactions in real time). These techniques will have a future impact on the development of point-of-care diagnostic devices applied for analyses of the Ca^{2+}-binding proteins in human diseases.

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SAR-by-NMR

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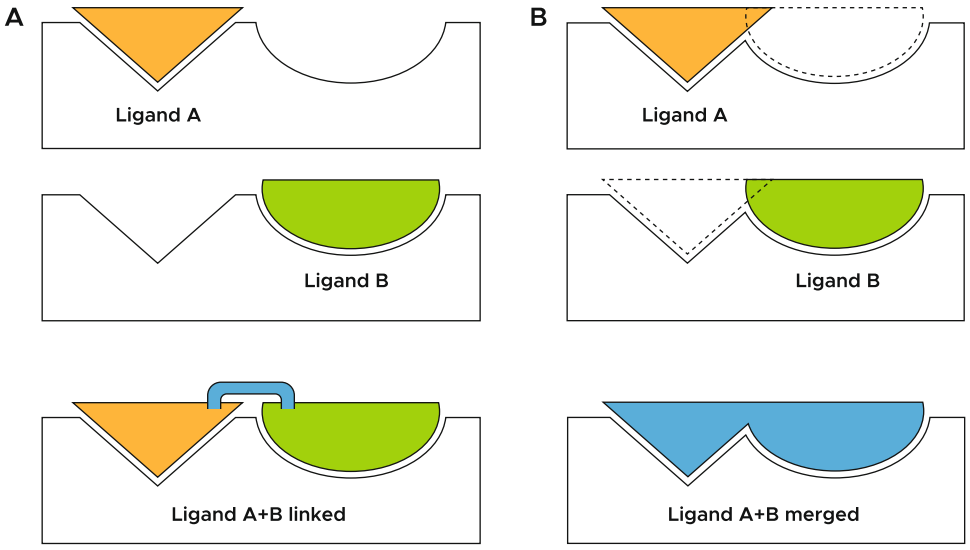
Synonyms

Fragment-based drug discovery; Lead discovery by NMR

Definition

Structure-activity-relationships (SAR)-by-NMR (Shuker et al. 1996) is originally the appellation of a method for generating systematically high-affinity small molecular weight protein ligands from small molecular fragments in the early stages of drug development. NMR is used for detecting weak protein-ligand interactions via chemical shift changes, usually monitored by $^{1}H–^{15}N$ correlation spectroscopy for amide proton and nitrogen signals (Shuker et al. 1996). Such chemical shift perturbations of individual backbone amide signals indicate also the protein binding site. The three-dimensional structure of a protein-ligand complex involving two ligands A and B simultaneously binding at adjacent sites is the basis for the design of a linker between the two fragments (Fig. 1a). This "linking" strategy produces a much tighter binding ligand from weakly binding fragments due to the saving of one entropic factor. In this procedure, protein-ligand interactions are detected via the protein NMR resonances, enabling the measurement or estimation of binding constants and a mapping of the binding site to detect suitably different locations for ligands to be linked.

Over time, SAR-by-NMR became an important element of fragment-based drug design approaches (Erlanson et al. 2016; Ribeiro de Souza Neto et al. 2020), in particular since low-affinity binding in the μM–mM range (as mostly occurring for fragments with a molecular weight between 150 and 300 Da) can be easily detected without creating too many "false positives." It is usually applied in concert with other biophysical techniques including structure determination methods. In this context, different experimental NMR approaches are also often used, detecting effects on the ligand NMR



SAR-by-NMR, Fig. 1 Schematics of SAR-by-NMR linking and merging procedures

spectrum (ligand-observed) rather than site-specific chemical shift changes of protein signals (protein-observed) (Gossert and Jahnke 2016). NMR techniques such as the saturation transfer difference (STD) and waterLOGSY experiments (Gossert and Jahnke 2016) observe ligand resonances to qualitatively detect binding, useful in screening campaigns or for hit validation. The path toward higher affinity is also often modified. Since the linking procedure is not always feasible, fragments A and B with overlapping binding sites may be improved by "merging" as depicted in Fig. 1b. In practice, individual fragments are often developed further by "growing," adding stepwise functional groups.

Basic Characteristics

The original SAR-by-NMR procedure (Fig. 1a) included searches for two fragment-like compounds (MW<250 Da) that bind close to each other near the active center and the structure-guided design of a linker to produce a tighter binding ligand (Shuker et al. 1996), characterized by the following steps:

- Screening of a compound library consisting of fragments in the molecular weight range of 150–250 Da to detect compounds that bind close to the active site of the protein, using correlation spectroscopy for detection
- Screening of derivatives of the initial hits, optionally supported by synthesis of a focused library yielding such derivatives to obtain compounds with improved affinity
- Screening for a second compound that binds in the vicinity of the first one using the same procedure
- Determination of a three-dimensional protein structure with both ligands bound
- Design of a linker for both ligands and synthesis of this compound
- Further improvement by synthesis of derivatives on the basis of the design

Key to the scheme above is the structure-based design of a linker. With its effect accounted for by ΔG_{link}, linking two fragments A and B produces a gain in affinity described by:

$$\Delta G_{AB} = \Delta G_A + \Delta G_B + \Delta G_{link}$$

The binding constant of the compound AB obtained after linking is then:

$$K_d(AB) = K_d(A) \times K_d(B) \times L,$$

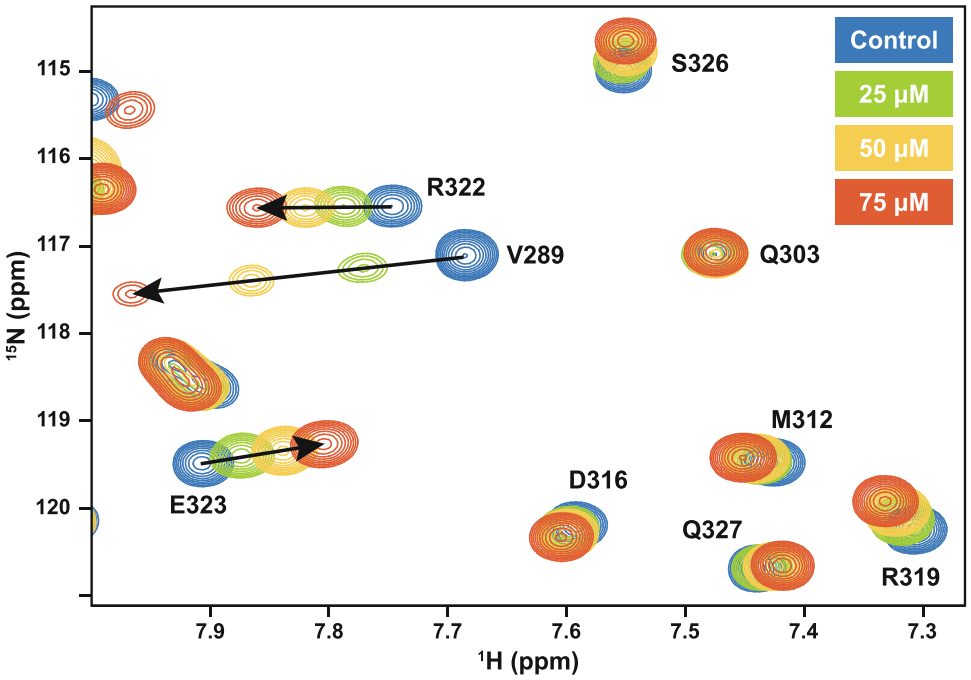
where L is the linking coefficient derived from ΔG_{link}.

In its modified forms (merging, growing), the first two steps of the original procedure are applied to obtain good starting points for choosing larger or merged compounds.

In current applications, between 500 and 10.000 substances are screened to find ligands binding in the mM–nM range. Often, a pre-selection by virtual screening approaches based on existing structural information is applied.

The methodology is often applied in a modified manner. Three-dimensional structures of complexes may also be obtained by X-ray crystallography or by molecular modelling making use of the detected chemical shift changes. The latter is often sufficient when individual hits of the screening phase are stepwise extended toward a tight-binding one. Due to the weak binding of fragments, and possible crystal packing effects, X-ray structures of complexes are sometimes difficult to obtain, requesting alternative methods.

An example for the initial NMR screening step is shown in Fig. 2. A region of a ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) shows the overlay of four different spectra obtained on ^{15}N-labelled dishevelled PDZ domain samples with four different ligand concentrations (0 mM, 25 mM, 50 mM, and 75 mM). The black NH signals are characteristic for the free protein, and the red signal is close to the chemical shift of the same signal from the complex. Only for a protein signal from an amino acid residue in the binding site, a journey from the signal position of the free to the equivalent of the liganded form may be observed (see R322, V289, and E323). For all other residues, signals in the four different spectra superimpose well within



SAR-by-NMR, Fig. 2 Superposition of regions from ^{1}H–^{15}N correlation spectra of the PDZ domain from the protein dishevelled obtained at different ligand concentrations. Chemical shift changes are indicated by arrows. Each cross peak originates from one particular amide group, as indicated by the amino acid one-letter code and its position in the protein sequence

certain limits, indicating that the chemical environment of the amide protons and nitrogen atoms did not change much through binding of the ligand or induced long-range structural effects.

In the example above, the effects of ligand binding were observed as gradual, concentration-dependent ^{1}H and ^{15}N chemical shift changes which are usually true for weak interactions with small molecular fragments below 300 Da. In general, two different effects may be observed depending on the binding constants and the chemical shift difference between the monitored signal of the complex and the signal of the free protein. At low fragment affinity, one travelling signal is observed that represents the weighted average of the chemical shifts of complex and free form of the protein. In the case of tight binding (nanomolar range and below) and large chemical shift differences, the signal of the free protein may decrease with increasing ligand concentration, and the signal of the complex will appear at its genuine chemical shift. These situations are called "fast exchange" (for the travelling signal) and "slow exchange" regimes. For intermediate cases, the signal may vanish at a certain point, called "coalescence."

The chemical shift changes observed in correlation spectra (the length of the vector in Fig. 2) may be taken directly as a measure of affinity differences when the effects of several ligands are compared. Such chemical shift (δ) changes (Δδ) are then quantified as a linear combination of proton (δ_H) and nitrogen (δ_N) shift contributions (Bertini et al. 2011).

$$\Delta\delta = \sqrt{\frac{(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2}{2}}$$

This provides also a basis for the measurement of binding constants. The dissociation constant may be determined by fitting the observed chemical shift change with increasing ligand concentration, up to very large excess (Gossert and Jahnke 2016).

Early examples of the application of SAR-by-NMR include the design of stromelysin and human papillomavirus E2 protein inhibitors (Hajduk et al. 1997a, b).

A detailed analysis of designed inhibitors for stromelysin by thermodynamical methods (Hajduk et al. 1997a) showed that the combination of two ligands, a biphenyl derivative and acetohydroxamic acid, yield an increase in the enthalpic contributions to the binding energy, whereas the entropic factor remained constant. In the application of the method to the design of ligands for the virus E2 protein (Hajduk et al. 1997b), the potential of NMR to detect low-affinity binding was exploited by starting a ligand refinement from compounds that bound in the millimolar range and combining features of two compounds to one which finally showed an IC_{50} of 10 micromolar in the applied test.

With fragment-based drug design developing into a coherent methodology, making use of many techniques, NMR contributes in several aspects but dominantly with its feature of detecting low-affinity binding and concomitantly the binding site. This is outlined in an analysis of a large number of fragment-based drug design studies by Mortenson et al. and Erlanson et al. for the years 2017 and 2018, respectively (Mortenson et al. 2019; Erlanson et al. 2020).

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Scaffold

► Adaptor Proteins

Second-Generation Antipsychotics

► Atypical Antipsychotics

Sedation

► GABAergic System

sEH

► Soluble Epoxide Hydrolase

Seminiferous Epithelial Barrier

► Blood-Testis Barrier

Senescence

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Synonyms

Cellular senescence

Definition

The term senescence originates from the Latin word *senesce*, meaning "to grow old," and was used for many years to allocate both cellular and organismal senescence (aging). Nowadays it is well established that at the systematic level, senescence is a hallmark of aging, the latter representing the gradual functional decline of proper organismal processes with age. At the cellular level, the terms "senescent" and "aged" are not identical and should not be used interchangeably (López-Otín et al. 2013; Gorgoulis et al. 2019, Table 1). According to the recently proposed consensus definition by the International Cell Senescence Association (ICSA), senescence (from now on used for cellular senescence) is ***a cellular state characterized by prolonged and generally irreversible cell-cycle arrest with secretory features, macromolecular damage, and altered metabolism that is triggered by stressful insults and in certain instances by physiological processes*** (Gorgoulis et al. 2019).

Basic Mechanisms

Inducers

Any stressor that challenges and compromises cellular homeostasis – particularly genome and/or proteome integrity – can potentially induce the senescent phenotype, if the intensity and the duration of the noxious (yet still sublethal) stimuli exceed a specific threshold which overcomes cell's repair capacity (Gorgoulis et al. 2018, 2019). So far myriads of intrinsic and extrinsic senescence inducers have been reported, including, but not limited to, telomere erosion/dysfunction, diverse genotoxic insults, oncogenes, epigenetic modifiers, and oxidative stress/metabolic imbalance stemming from proper protein turnover impairment, mitochondrial dysfunction, and ribosomal and endoplasmic reticulum stress (Gorgoulis et al. 2018, 2019; Myrianthopoulos et al. 2019).

Hallmarks, Molecular Pathways, Markers

Senescence is appreciated as a stress response mechanism characterized by the following **main** interconnected hallmarks: **(1)** ***prolonged and generally irreversible cell-cycle arrest***, mediated predominantly by two signaling pathways that act in a temporal manner: that of p53-p21$^{WAF1/Cip1}$ and Rb-p16^{INK4a} (Gorgoulis et al. 2018, 2019; Myrianthopoulos et al. 2019). The senescence-associated cell cycle withdrawal is not universally an irreversible program as it is currently well established that in certain settings senescent cells can "escape" from the arrested state and re-enter the cell cycle (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019; Galanos et al. 2016; Komseli et al. 2018). **(2)** autocrine or paracrine **secretory properties** termed ***senescence associated secretory phenotype (SASP)***. The senescence secretome consists of cytokines, chemokines, growth factors, proteases, and other factors, depending on the nature of the stressor, cell type, and senescence endurance (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). This feature exerts immunomodulatory functions that affect the tissue microenvironment and is controlled by various signalling cascades [e.g., mammalian target of rapamycin (mTOR), mitogen-

Senescence, Table 1 Differences between senescence and aging

Senescence	Aging
Well-orchestrated response	Unprogrammed, random process
Although the initial factors which trigger senescence (e.g., DNA damage) may itself be random, the accompanying cellular changes associated with cell senescence are not random, but a rather orchestrated response	Results from the accumulation of random(stochastic) damage leading to impairment in cell function with time
Senescence occurs throughout the lifespan, including during embryogenesis. Cells of any age can undergo senescence	Time (age)-dependent functional decline
Can be triggered not only by telomere attrition (which is definitely related to age), but also by nontelomeric, not aging-associated, acute genotoxic insults (stress-induced senescence)	Telomere attrition is a hallmark of aging, but other acute stimuli cannot elicit a specific aged cell phenotype
Cell-cycle arrested in G1, G1/S, or G2, depending on the activated checkpoint	May not be cell-cycle arrested??
It can be considered as a "sudden" phenomenon meaning that whatever the trigger (telomeric or nontelomeric) or the duration of the phenomenon, the establishment of the cell-cycle arrest coupled to phenotypic modifications is a rather immediate response, induced within relatively short periods of times	The loss of physiological cellular integrity that characterizes aged cell is gradual and progressive, not an "on-off" phenomenon
Both detrimental and beneficial depending on the biological context	Detrimental to the function of normal biological processes
Reversible ("escape" from senescence)	Terminal, permanent, nonreversible phenomenon (regeneration?)
Hyper-secretory phenotype, hyperfunction	–
SA-β-gal positive	?

activated protein kinase (MAPK)-p38, phosphoinositide 3 kinase (PI3K)/AKT, Notch1, and NOTCH/JAG1], eventually leading to NF-κB and CCAAT/enhancer binding protein beta (C/EBPβ) transcriptional upregulation. Cytoplasmic bridges and extracellular vesicles are also involved (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). **(3)** ***macromolecular damage***, in the form of DNA, protein, and lipid damage, as a result of decline in quality control and damaged turnover mechanisms, commonly evident in the stressful environment of senescence (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). Of note, macromolecular damage is a unique feature of senescent cells allowing their distinction from other non-dividing cells, such as quiescent or terminally differentiated cells (Gorgoulis et al. 2019). DNA damage, mainly in the form of persistent DNA damage foci, is the most well-established type of macromolecular damage occurring in most types of senescence (for instance, in developmental senescence DNA damage and DDR are absent). Persistent DNA damage foci include irreparable lesions principally related to telomeric DNA (TIFs, telomere dysfunction-induced foci; TAFs, telomere-associated foci) and DNA-SCARSs (DNA segments with chromatin alterations reinforcing senescence). DNA damage elicits the activation of the DNA damage response pathway (DDR), which in the case of oncogene-induced senescence (OIS) cooperates with members of the *CDKN2A* locus (*ARF*) to exert anti-tumor functions (Gorgoulis et al. 2018, 2019; Myrianthopoulos et al. 2019; Evangelou et al. 2013; Velimezi et al. 2013). Cytoplasmic chromatin fragments (CCFs), another form of DNA damage in the senescence context, are related to the activation of a proinflammatory reaction by the cGAS-cGAMP-STING network. Regarding protein and lipid damage, they are the result of damaged biomolecule accumulation due to excessive reactive oxygen species (ROS)

production and improper degradation (Gorgoulis et al. 2018, 2019; Myrianthopoulos et al. 2019; Vasileiou et al. 2019). **(4)** ***Altered metabolism***, related to (i) changes in mitochondrial function, dynamics, and morphology. Mitochondria appear abundant but dysfunctional, producing increased ROS levels and (ii) lysosomal aggregation and malfunction, associated with elevated senescence-associated b-galactosidase (SA-b-Gal) activity and intralysosomal accumulation of lipofuscin (Georgakopoulou et al. 2013; Evangelou et al. 2017). Altogether, aggregation within senescent cells of large dysfunctional mitochondria and lysosomes, the latter enriched in lipofuscin granules, is reflected by increased cytoplasmic granularity (Gorgoulis et al. 2019; Vasileiou et al. 2019).

Another potent property of senescent cells is their ***resistance to apoptosis***, and inhibition of the BCL-2 cell death regulator family of proteins has been exploited in senolytic strategies. The BCL-2 family consists of both pro-apoptotic and anti-apoptotic factors. Among anti-apoptotic ones are BCL-2, BCL-XL, BCL-w, BCL-2-related protein A1 BFL-1 (A1), myeloid cell leukemia 1 (MCL-1), and BCL-B/Boo, featuring four BCL-2 homology (BH) domains (BH1, BH2, BH3, and BH4). The pro-apoptotic fraction includes the multi-domain pro-apoptotic effectors, such as BAK and BAX and the so-called "BH3-only proteins" such as BAD, BID, BIK, BIM, BMF, HRK, PUMA, and NOXA, which harbor a short BH3 domain (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). Depending on the initial stressor/signal and cell type, senescent cells can acquire numerous additional salient cellular and subcellular features (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019) related to: ***(i) morphological aspects***, such as cellular flattening/elongation, irregular shape, and enlargement due to cytoskeleton rearrangements and alterations in plasma membrane composition and increase in nuclear and nucleolar size as well as fragmentation of the nuclei (polyploidy), mitochondria, and endoplasmic reticulum; ***(ii) epigenetic traits*** that include chromatin modifications (e.g., elevated H4K20me3 and H3K9me, H4K16ac, H3K27me3 "canyons," H3K4me3 "mesas," senescence-associated distension of satellites (SADSs), senescence-associated heterochromatin foci (SAFHs), and others), accompanied by characteristic transcriptional signatures, miRNAs, and non-coding RNA expression; and ***(iii) protein markers*** that are either present (DCR2, NKG2D ligands) or absent (for instance, proliferation markers, Lamin B1). For the time being, none of the above-described markers (stemming from features and signaling pathways) is sufficient alone for the identification of senescent cells with absolute specificity. Based on the fact that some markers have a broader spectrum while others depict specific senescence types, the ICSA consortium has proposed a specific multi-marker approach for robust detection (Gorgoulis et al. 2019) that allows accurate estimation of the effectiveness of the anti-senescence strategies.

Physiological and Pathological Implications

Senescence could be metaphorically described as the Greek god Hermathena or the Roman god Janus both facing to two opposite directions depending on the situation. Of clinical importance, short-term induction of senescence has a favorable outcome during development (*developmentally programmed or embryonic senescence*) and in normal processes, like tissue repair *(acute senescence*) (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). Additionally, cellular senescence plays a crucial role as an anti-tumor barrier at the early stages of tumorigenesis, preventing neoplastic cells from further expansion and propagation (Gorgoulis et al. 2019; Bartkova et al. 2006; Halazonetis et al. 2008; Gorgoulis and Halazonetis 2010). On the other hand, persistence of senescence is associated with harmful properties mediated mainly via paracrine and/or systematic SASP, while escape from OIS is implicated in tumor progression and remission (Galanos et al. 2016; Komseli et al. 2018). Accumulation of senescent cells results from chronic stress damage and/or insufficient clearance by the immune system (*chronic senescence*). As a consequence, not only the regenerative potential and function of tissues/organs are impaired, but also a "slow burning" inflammatory milieu is maintained, triggering the

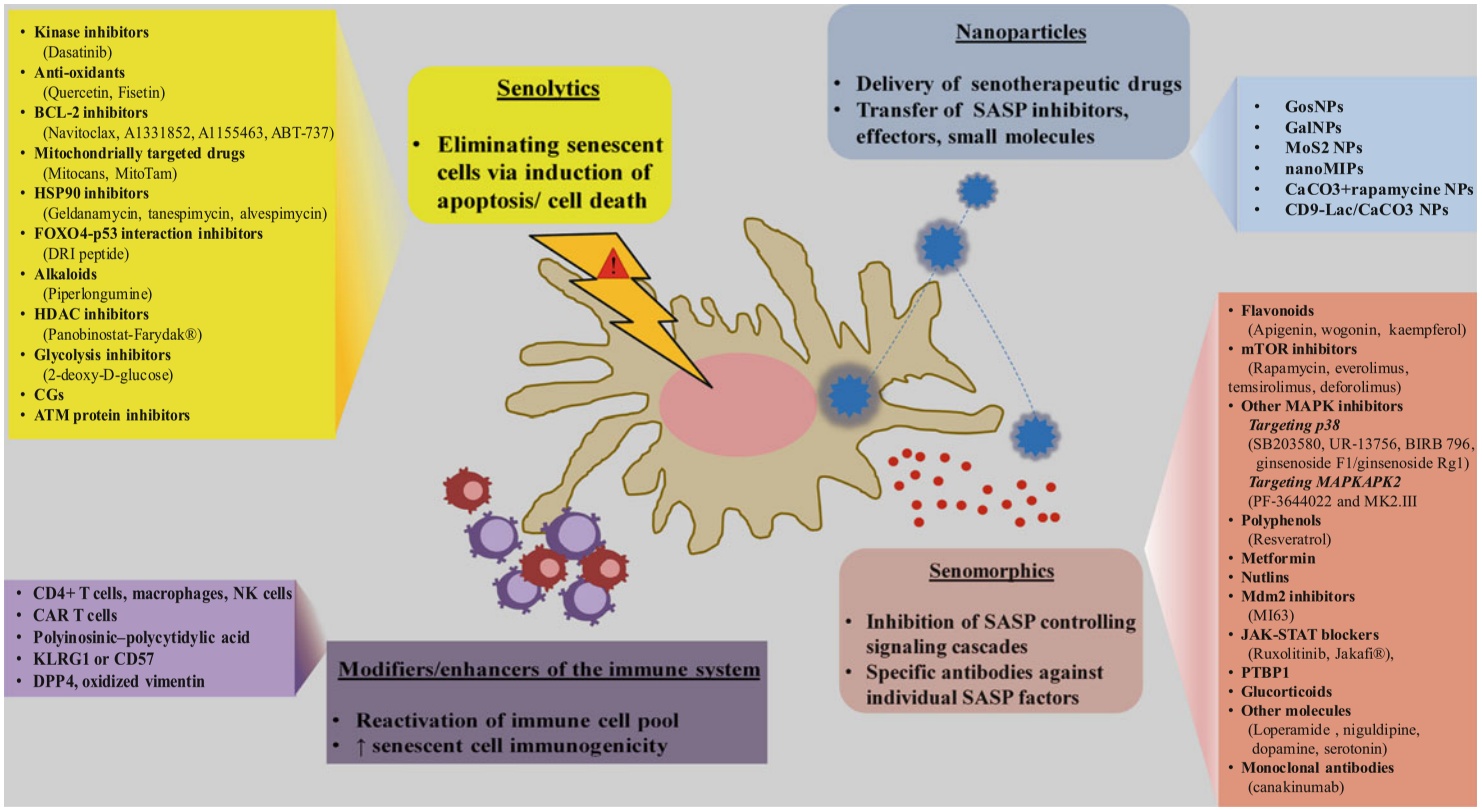
aging process and various pathological conditions such as cardiovascular, kidney, liver, and neurodegenerative diseases, cancer, type 2 diabetes, osteoarthritis, cataract, idiopathic pulmonary fibrosis, metabolic syndrome, and cahexia (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019; Munoz-Espin and Serrano 2014).

Pharmacological (Therapeutic) Intervention

Given the continuously growing knowledge on the wide spectrum of adverse effects of chronic senescence, as already mentioned, the development of new therapies that selectively target senescent cells or neutralize their undesirable properties emerged as an attractive challenge. Research on senotherapeutic agents and their precise mode of action (molecular key players, pathways, and mechanisms, Fig. 1), although quite restricted, is rapidly expanding, and this information is currently exploited for clinical testing (Table 2). This exciting and relatively new field encompassing molecular and chemical biology and pharmaceutical chemistry along with sophisticated methodologies and advanced technologies (e.g., efficient process of "big data" via machine learning) for drug discovery (Sakellaropoulos et al. 2019; Vougas et al. 2019) has already provide aspects of its potential and future therapeutic opportunities. For the time being, the vast majority of available senotherapeutic compounds are either drugs already used for other research and clinical purposes or extensively investigated natural derivatives. Traditional and next-generation, direct or indirect, senotherapies are based on the following classes of molecules: ***(1) senolytics***, ***(2) senomorphics or senostatics*** (SASP inhibitors), ***(3) modifiers/enhancers of the immune system,*** and ***(4) nanoparticles***.

Senolytics

Senolytics are molecules that impede cardinal senescence pro-survival pathways rendering senescent cells vulnerable to cell death. The first described compounds with senolytic properties were dasatinib and quercetin (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Dasatinib is a kinase inhibitor that was traditionally used as an anticancer drug. Apart from the activity of various protein tyrosine kinases such as BCR/Abl, c-Kit, and Scr family members, dasatinib blocks Ephrin receptors, as well as promotes apoptosis. These receptors interact with two ligands known as Ephrin 1 (EFNB1) and Ephrin 3 (EFNB3) that have been shown to be upregulated in senescent cells, generating thus critical pro-survival (anti-apoptotic) signals. Quercetin, in turn, is a flavonoid with well-known antioxidant properties that can modulate various molecular cascades such as the NF-kB, PI3K/AKT, mTOR, p53/p21$^{WAF/CIP1}$, serpin, and estrogen receptor signaling pathways (Gorgoulis et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). Regarding senolysis by quercetin, inhibition of the PI3Kδ isoform has been favored as the most prevalent mechanism for apoptosis induction. Administration of dasatinib or quercetin has been reported to effectively but variably, depending on the cell type, alleviate aging and senescence *in vitro* and *in vivo* (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Interestingly, their combination exerts a broader senolytic outcome and has been demonstrated in clinical trials to effectively reduce the senescent cell "load" in humans (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019, Table 2). Among other recently demonstrated flavonoids exerting senolytic efficiency is fisetin that behaves similarly to quercetin, as expected by their slight structural differences (hydroxyl group at position 5 of the chromone scaffold). Fisetin affects a variety of cellular functions as well as molecular key players/pathways including topoisomerases; cyclin-dependent kinases; the NF-kB, PI3K/AKT/mTOR, PPAR, and PARP1 signaling pathways; and epigenetic modifiers (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). Based on such a multilevel activity, its several beneficial effects, for instance, on cancer prevention and neurodegenerative processes, seem to rely not only on the antioxidant potential but also on their impact on senescence and SASP, a feature that seems valid for the majority of flavonoids in promoting well-being and health.



Senescence, Fig. 1 Schematic overview of direct and indirect senotherapeutic approaches and corresponding drugs/agents currently applied or proposed (for details see text)

Senescence, Table 2 Senotherapeutics in clinical trials

Drug-drug combination	Mechanism of action	Clinical trial Id	Clinical entity under investigation	Clinical trial phase
Senolytics				
Dasatinib + quercetin	PI3K-AKT pathway inhibitor	NCT02848131	Chronic kidney disease	
		NCT02874989	Idiopathic pulmonary fibrosis	
		NCT02652052	Hematopoietic stem cell transplant survivors	
		NCT04063124	Alzheimer's	Phase 1 and 2
Navitoclax + cisplatin and etoposide	Bcl-2 inhibitor	NCT00878449	Small cell lung cancer (SCLC)	Phase 1 and 1/2
Navitoclax + dabrafenib and trametinib	B-RAF inhibitor and MEK inhibitor	NCT01989585	BRAF mutant metastatic melanoma Stage III–IV cutaneous melanoma AJCC v7 Unresectable melanoma	Phase 1 and 1/2
Navitoclax + osimertinib	Tyrosine kinase inhibitor acting on EGFR (T790M + mutation)	NCT02520778	EGFR mutant-positive advanced NSCLC (previously treated with another EGFR inhibitor, including metastatic cancer) Stage III–IV lung non-small cell cancer AJCC v7	Phase 1 and 1/2
Navitoclax + gemcitabine	Nucleoside analogue	NCT00887757	Advanced solid tumors	N/A
Navitoclax + paclitaxel	Microtubule inhibitor (block depolymerization of microtubules)	NCT00891605	Advanced solid tumors	Phase 1
Navitoclax + docetaxel	Microtubule inhibitor (block depolymerization of microtubules)	NCT00888108	Advanced solid tumors	Phase 1
Navitoclax + irinotecan	Topoisomerase 1 inhibitor	NCT01009073	Advanced solid tumors	Phase 1
Navitoclax + erlotinib	Tyrosine kinase inhibitor acting on EGFR	NCT01009073	Advanced solid tumors	Phase 1
Navitoclax + sorafenib	Protein kinase inhibitor of VEGFR, PDGFR, RAF kinases (c-RAF > B-RAF)	NCT02143401	Advanced solid tumors (relapsed or refractory) Metastatic malignant solid neoplasm Recurrent hepatocellular carcinoma Recurrent malignant solid neoplasm Refractory malignant neoplasm Stage IV hepatocellular carcinoma AJCC v7 Unresectable solid neoplasm	Phase 1
Navitoclax + trametinib	MEK inhibitor (MAPK/ERK pathway inhibitors, both MEK1 and MEK2)	NCT02079740	KRAS or NRAS mutation-positive advanced solid tumors (including metastasis) Advanced lung carcinoma Advanced malignant solid neoplasm Advanced pancreatic carcinoma Malignant female reproductive system neoplasm Metastatic malignant solid neoplasm	Phase 1b and 2

(continued)

Senescence, Table 2 (continued)

Drug-drug combination	Mechanism of action	Clinical trial Id	Clinical entity under investigation	Clinical trial phase
			Recurrent lung carcinoma Recurrent malignant solid neoplasm Recurrent pancreatic carcinoma Stage III–IV lung cancer AJCC v7 Stage III–IV pancreatic cancer AJCC v6 and v7 Unresectable malignant solid neoplasm	
Leniolisib (CDZ173)	Selective PI3Kδ inhibitor	NCT02435173	CVID: common variable immunodeficiency KRAS APDS: Activated PI3K-delta syndrome Advanced Lung PASLI: p110 delta activating mutation causing senescent T cells, lymphadenopathy, and immunodeficiency	Phase 2 and 3
		NCT02859727	Extension to the study above (patients with genetically activated PI3Kδ carcinoma-APDS/PASLI)	Phase 2 and 3
Metformin	mTOR inhibitor	NCT03309007	Pre-diabetes in the elderly	Phase 3
Fisetin	PI3K/AKT and ROS inhibitor	NCT04210986	Osteoarthritis, knee	Phase 1 and 2
Senomorphics				
Metformin	mTOR inhibitor, IKK/NF-κB inhibitor	NCT03309007	Pre-diabetes in the elderly	Phase 3
		NCT03451006	Aging, inflammation, frailty	Phase 2
Topical rapamycin (sirolimus)	mTOR inhibitor	NCT03103893	Human skin senescence	Completed
Rapamycin (sirolimus)		NCT01649960	Coronary artery disease	Phase2

Another potent group of senolytic drugs includes first- and second-generation inhibitors of the BCL-2 (B CELL LYMPHOMA-2) cell death regulator family of proteins, implicated in triggering the intrinsic mitochondrial apoptosis pathway. Navitoclax (ABT263), an anticancer drug that specifically targets BCL-2, BCL-W, and BCL-XL, was the first described inhibitor of the BCL-2 family with senolytic properties (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). The molecule behaves as a protein-protein interaction inhibitor (PPI), disrupting the interaction between BCL-2 proteins and their endogenous inhibitory regulators (BH3 domain-containing proteins). Despite the severe side effects (platelet and neutrophil toxicity) that rendered clinical testing unfeasible, establishment of Navitoclax as a senolytic opened new horizons for the identification of other novel selective BCL-XL inhibitors that simultaneously eliminate senescent cells. Indeed, additional such compounds entered the scenery. A1331852 and A1155463 are members of the flavonoid family that were initially applied for cancer treatment (Paez-Ribes et al. 2019; von Kobbe 2019). They are high-affinity BH3 mimetic ligands of BCL-XL and selectively disrupt the BCL-XL-BIM complex, exerting senolytic properties in a cell type-specific manner but with lower toxicity that renders them attractive candidates for clinical applications (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). An additional BH3 mimetic compound and PPI inhibitor, the anticancer drug ABT-737, binds to the hydrophobic groove of BCL-2, BCL-XL, and BCL-W in contrast to pro-survival proteins MCL-1, BCL-B,

and BFL-1 (A1) that are resistant. Additionally, mitocans (mitochondrially targeted anti-cancer drugs) are also BH3 mimetics known to induce apoptosis in malignant cells. Given that senescent cells exhibit an increased mitochondrial load, these compounds emerged as intriguing candidates for putative senolytic interventions. Interestingly, the mitochondria-targeted tamoxifen (MitoTam) has been reported to selectively eliminate senescent cells *in vitro* and *in vivo* (Paez-Ribes et al. 2019; von Kobbe 2019). The molecule impedes mitochondrial oxidative phosphorylation (OXPHOS) in senescent cells resulting in the loss of mitochondrial integrity and eventually apoptosis.

The wide range of senolytics also includes molecules that belong to the following subclasses (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019): **(i)** FOXO4-p53 interaction inhibitors (DRI peptide), **(ii)** alkaloids (piperlongumine and analogues), **(iii)** heat shock protein (HSP90) inhibitors, **(iv)** histone deacetylase (HDAC) inhibitors (Panobinostat-Farydak®), **(v)** glycolysis inhibitors (2-deoxy-D-glucose), **(vi)** cardiac glycosides (CGs), and **(vii)** ataxia-telangiectasia mutated (ATM) protein inhibitors. FOXO4 (Forkhead box protein O4), a member of the forkhead family transcription factors, interacts with p53 at sites of DNA damage, up regulating the senescence master regulator $p21^{WAF1/CIP1}$. This interaction can be disrupted by a FOXO4 D-retro-inverso (DRI) peptide that selectively competes with FOXO4 for p53 binding, causing p53 nuclear exclusion in senescent cells and thus promoting their apoptosis, as shown in both *in vitro* and *in vivo* settings (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Piperlongumine is a natural compound shown to induce apoptosis in cancer cells by generating reactive oxygen species (ROS) or through modulation of the NF-kB cascade. The latter along with the fact that the exact mechanism of senolytic activity remains still unraveled but seems independent from ROS production raises the possibility of a common behavior between alkaloids and flavonoids in eliminating senescent cells (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). HSP90 inhibitors include the natural antitumor antibiotic geldanamycin and its analogues tanespimycin (17-AAG) and alvespimycin (17-DMAG). They belong to the macrocyclic lactam class of agents and inhibit HSP90 through binding to the N-terminal ATP pocket. This inhibition seems to be associated with AKT (protein kinase b) destabilization and apoptosis induction, reminding the mode of action of other senolytics such as quercetin (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Interestingly, in contrast to other senolytics, their spectrum of efficacy is likely broader in terms of cell type affected. Among the HDAC inhibitors recently described as senolytics, panobinostat is the most prevalent compound. Panobinostat has been shown to exert a senolytic potential and a synergistic effect on persistent senescent cells after their treatment with taxol or cisplatin (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). At a different level, altered (mainly hypermetabolic) activity and elevated glycolysis commonly evident in senescent cells are essential for their survival. In the context of therapy-induced senescence or OIS, treatment of senescent cells with 2-deoxy-D-glucose (2-DG), a pseudo-substrate for glycolysis, renders them selectively susceptible to apoptosis (Paez-Ribes et al. 2019). Cardiac glycosides (CGs) impose a similar vulnerability to senescent cells by affecting cell membrane Na/K-ATPas and altering thus the electrochemical gradient balance. Lastly, KU-60019 an ATM and DDR inhibitor seems also to exert an effective anti-senescence potential in *in vivo* settings recapitulating age-related processes, via regulation of lysosomal acidification, removal of dysfunctional mitochondria, and metabolic reprogramming (Paez-Ribes et al. 2019).

Senomorphics or Senostatics (SASP Inhibitors)

Senomorphics or senostatics are agents that exert inhibitory effects on SASP functions and not on senescent cell viability itself. They include compounds that target various SASP controlling signalling cascades or specific antibodies against individual SASP factors such as IL-1b, IL-6,

and IL-8. Given the huge diversity of putative targets, the number and the spectrum of described senomorphic compounds are extended (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). Flavonoids such as apigenin, wogonin, or kaempferol mediate suppression of SASP components and particularly IL-1a, IL-1b, IL-6, IL-8, GM-CSF, CXCL1, monocyte chemoattractant protein-2 (MCP-2), and MMP-3, partially via the NF-kB p65 subunit and the NF-kB inhibitor IkBz (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Rapamycin (Rapamune®) has also been shown to exert senomorphic functions. This natural macrolide is a well-known selective inhibitor of the mTOR pathway. mTOR activates MAPKAPK2 (MAPK activated protein kinase 2) translation, resulting in phosphorylation of the member of the TIS11 family of early response proteins ZFP36L1 (ZFP36 ring finger protein like 1) and direct induction of various SASP components (Paez-Ribes et al. 2019; von Kobbe 2019). In addition, IL-1α translation inhibition and IL-6 downregulation by rapamycin can be exerted via an Nrf2-independent mechanism. Rapamycin is also known to regulate prelamin A, a factor implicated in longevity. Novel mTOR inhibitors such as everolimus, temsirolimus, and deforolimus exerting superior pharmacological properties have also entered the scenery of senomorphics (Paez-Ribes et al. 2019). Among other inhibitors of the MAPK pathway, those targeting p38 (SB203580, UR-13756, BIRB 796, and ginsenoside F1/ginsenoside Rg1) and MAPKAPK2 (PF-3644022 and MK2.III) have been demonstrated to suppress several SASP components (for instance, IL-6 and IL-8) by inhibiting NF-κB transcriptional activation and paracrine effects in human senescent cells (Paez-Ribes et al. 2019; von Kobbe 2019).

A different class of agents presenting senomorphic activity includes polyphenols and especially resveratrol, a natural trans-stilbene polyphenol with a wide spectrum of bioactivities. Regarding its senomorpic properties, resveratrol modulates potent cellular signaling pathways such as NF-kB (IkB inhibition) and PI3K/AKT, controlling thus the release of multiple pro-inflammatory cytokines (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). Resveratrol also activates SIRT1, a class III histone deacetylase, the former with notorious properties in lifespan and AMPK (AMP-activated protein kinase) and the latter implicated in various processes including metabolism and aging. In addition, the molecule and its synthetic analogues (polar substitution on the 4' position) can restore the expression of mRNA splicing factors, and therefore these agents have been proposed as potential drugs for reversing the senescent phenotype (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). This function was independent of SIRT1 activation and most probably related to enhanced telomere maintenance.

The intriguing beneficial impact of the antidiabetic drug metformin (Glucophage®) on aging and life span expansion led to initiation of drug repurposing research seeking for its putative role on senescence. These efforts revealed some targets/levels of senescence regulation by metformin (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019): ***(i)*** decrease inNF-κB nuclear translocation and inhibition of phosphorylation of the two catalytic subunits of IkB kinase, namely, IKK α and β, leading to downregulation of several SASP factors, namely, CXCL-5, IL-6, IL-8, and IL-1β; ***(ii)*** AMPKa stimulation causing mTOR pathway inhibition; ***(iii)*** tuning of mitochondrial electron transport, known to eventually affect regulation of pro-inflammatory cytokines such as IL-6; and ***(iv)*** mitochondrial glycerol-3-phosphate dehydrogenase (GPDH) and lysine-specific demethylase 6A (KDM6A/UTX) control. Based on the aforementioned beneficial effects on health and life span, testing for additional therapeutic opportunities in the frame of clinical trials is currently ongoing (Targeting Aging with MEtformin – TAME study, Table 2).

Nutlins utilized for cancer treatment can impose apoptosis and/or senescence by inhibiting Mdm2 and stabilizing p53. However, these molecules apart from inducing senescence exert also some anti-senescence properties. Nutlin-3a is known to suppress NF-κB in a p53-dependent manner, driving downregulation of certain SASP interleukins in human cells (Paez-Ribes et al.

2019; von Kobbe 2019). A similar potential for SASP inhibition has been reported for the next-generation Mdm2 inhibitor MI-63 (Paez-Ribes et al. 2019). Blocking of the JAK/STAT pathway by targeting JAK kinases (ruxolitinib, Jakafi®), eventually leading to inactivation of C/EBPβ, has also been linked with SASP alleviation. Among the JAK kinases, blocking of JAK1/2 drives the decrease of the SASP modulator activin A that is frequently found upregulated during aging (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Likewise, NOTCH1 upregulation has been associated with SASP suppression, via C/EBPβ downregulation, in the context of OIS (Paez-Ribes et al. 2019). In addition, the alternative splicing modulator polypyrimidine tract binding protein 1 (PTBP1) has emerged as promising therapeutic target for SASP inhibition and is currently under investigation (Paez-Ribes et al. 2019). Another class of drugs harboring SASP inhibitory potential includes the glucocorticoids, steroid hormones with well-characterized anti-inflammatory properties (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). The most effective compounds for SASP (IL-1a and IL-6) suppression, via control of NF-kB transcription, are corticosterone and cortisol. In contrast, dexamethasone has been demonstrated to induce senescence via SIRT1 inhibition and activation of the $p53/p21^{WAF/CIP1}$ axis, proving a putative explanation for its adverse side effects. Notably, some other clinically applied drugs such as loperamide and niguldipine as well as dopamine and serotonin antagonists have been reported to exhibit senomorphic properties (Myrianthopoulos et al. 2019). Lastly, an additional way to directly target SASP components (ligands or receptors) encompasses the use of monoclonal antibodies. Canakinumab that reacts against IL-1b and has been extensively used to treat autoimmune diseases comprises such an example (Paez-Ribes et al. 2019).

Modifiers/Enhancers of the Immune System

The immune system undergoes an age-related decline that has been associated with accumulation of senescent cells in tissues. Given that senescent cells are immunogenic, stimulation of the immune system by immunotherapy, currently used for treatment of cancer and other diseases, has also emerged as an attractive strategy. Senescent cell clearance is achieved by interventions that aim at two levels: reactivation of the immune cell pool or increase in senescent cell immunogenicity. Senescent cells are eliminated by $CD4^+$ T cells, macrophages, and NK cells (senescence surveillance). Chimeric antigen receptor (CAR) T cells against senescence occurring agents, similar to those developed for cancer therapies, seem a promising choice (Paez-Ribes et al. 2019; von Kobbe 2019). Moreover, restoration of T cell and NK functions that decline during the development of age-related pathologies and aging can be achieved via inhibition of AMPK by an p38 MAPK inhibitor or via downregulation of the killer cell lectin-like receptor G1 (KLRG1 or CD57) (Paez-Ribes et al. 2019; von Kobbe 2019). Treatment with polyinosinic-polycytidylic acid, a NK toll-like receptor 3 (TLR3) ligand and interferon-γ, increases in certain settings the cytotoxic activity of NK cells via the NK cell receptor NKG2D (Paez-Ribes et al. 2019; von Kobbe 2019). The latter interacts with corresponding ligands frequently upregulated in senescent cells. As for enhancing senescent cell immunogenicity, the surface marker DPP4 can be exploited in the context of an antibody-dependent NK cell-mediated cytotoxicity, for senescent cell elimination (Paez-Ribes et al. 2019; von Kobbe 2019). Similarly, an oxidized form of membrane-bound vimentin discovered in senescent fibroblasts could be recognized and targeted by humoral innate immunity processes (Paez-Ribes et al. 2019).

Nanoparticles

Nanoparticles (NPs) are not considered in a strict manner, as agents that can alone influence the viability or the function of senescent cells. In fact, NPs behave as carriers, able to efficiently deliver among other substrates, a senotherapeutic drug for targeted clearance or manipulation of the deleterious potential of the senescent cell. In this context, mesoporous silica nanoparticles capped with a mixture of galacto-oligosaccharides (GosNPs) of different lengths were the pioneer cargo delivery system

generated. GosNPs enter cells via endocytosis and fuse with lysosomes that in senescent cells are associated with elevated endogenous SA-βgal activity (Paez-Ribes et al. 2019). This interaction results in enzymatic hydrolysis of the cap and release of the cargo selectively in senescent cells. Subsequently, silica beads capped with 6-mer galacto-oligosaccharides (GalNPs) have been reported as superior mediators for effective and specific (with lower toxicity) transport of drugs in *in vivo* models of damage- and chemotherapy-induced senescence (Paez-Ribes et al. 2019). Such evidence opens new perspectives in enhancing the beneficial effect of chemotherapy by elimination of cancer cells undergoing chemotherapy-induced senescence. Moreover, molybdenum disulfide nanoparticles (MoS2 NPs) as well as molecularly imprinted nanoparticles (nanoMIPs) have been generated and tested *in vitro* (Paez-Ribes et al. 2019). MoS2 NPs seem to exhibit low preference for senescent cells. In contrast, nanoMIPs loaded with dasatinib can eliminate senescent cells by internalizing the drug, after the recognition of surface molecules such as b2 microglobulin (B2M) (Paez-Ribes et al. 2019).

Alternatively, other emerging nanovehicles aim to restrain SASP by transferring encapsulated inhibitors, effectors, and small molecules. Examples applied and tested in *in vitro* settings but still lacking in vivo evidence include (Paez-Ribes et al. 2019) (i) rapamycin containing porous calcium carbonate nanoparticles (CaCO3). These NPs are coated with a conjugate of lactose (Lac, galactose/glucose dimers linked with β-1,4- glycosidic units) that allows cargo release in the presence of increased SAβ-gal activity and polyethylene glycol imposing stability in the blood and opsonisation avoidance. (ii) The latter nanodevices, conjugated with a monoclonal antibody against CD9. CD9-Lac/CaCO3 exerts their delivery properties by interacting with CD9, commonly located at the surface senescent cells that simultaneously exhibit elevated SAβ-gal activity, thus strengthening cell specific uptake and cargo release.

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Conflict of Interest The authors wish to declare no conflict of interest.

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Sepsis

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Synonyms

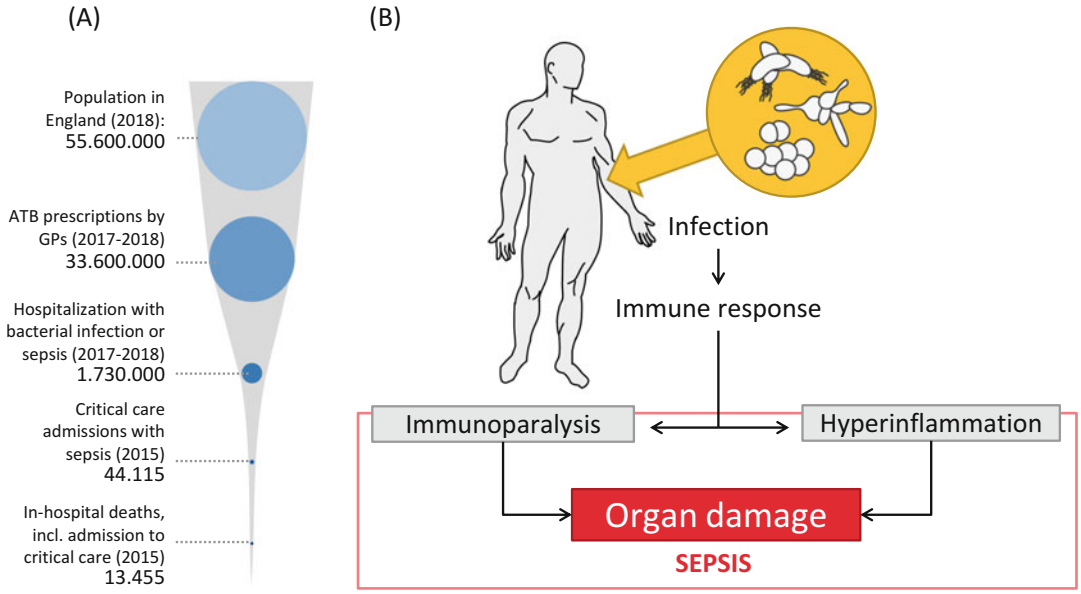
Blood poisoning; Infection; Organ dysfunction; Systemic inflammation; Septic shock

Definition

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Organ dysfunction is the criterion that distinguishes sepsis from minor, self-limiting infections (Singer et al. 2016). Main clinical manifestations are altered mentation, high respiratory rates, and low blood pressure that occur in patients with suspected infection (Seymour et al. 2016).

Basic Mechanisms

Sepsis has long been interpreted as an excessive systemic inflammatory response due to infection ("systemic inflammatory response syndrome, SIRS"; Bone et al. 1992). Failing containment of invading pathogens and pathologic hyperinflammatory immune responses were thought causative of impaired organ function. The exclusive reliance on SIRS as the hallmark of sepsis has been challenged by the finding that SIRS criteria are absent in a significant proportion of patients (Kaukonen et al. 2015). Moreover, reduced immune responsiveness ("immunoparalysis") has been shown to dominate sepsis pathology, at least in later stages of the disease process (Hotchkiss et al. 2013). These opposing response patterns are both able to provoke remote organ



Sepsis, Fig. 1 Dysfunctional immune responses in sepsis (**a**) Infection is a frequent and typically self-limiting event. Approximately 2.5% of patients presenting in hospitals with infection will be admitted to an intensive care unit due to infection-triggered organ failure, i.e., sepsis (data adapted from (Singer et al. 2019), circle areas are proportional to population and case numbers). (**b**) Organ damage in sepsis can be provoked by either reduced immune responsiveness (immunoparalysis) or hyper-inflammatory responses to infections (immunopathology) in a minority of patients. Abbreviations: *ATB* antibiotic, *GP* general practitioner

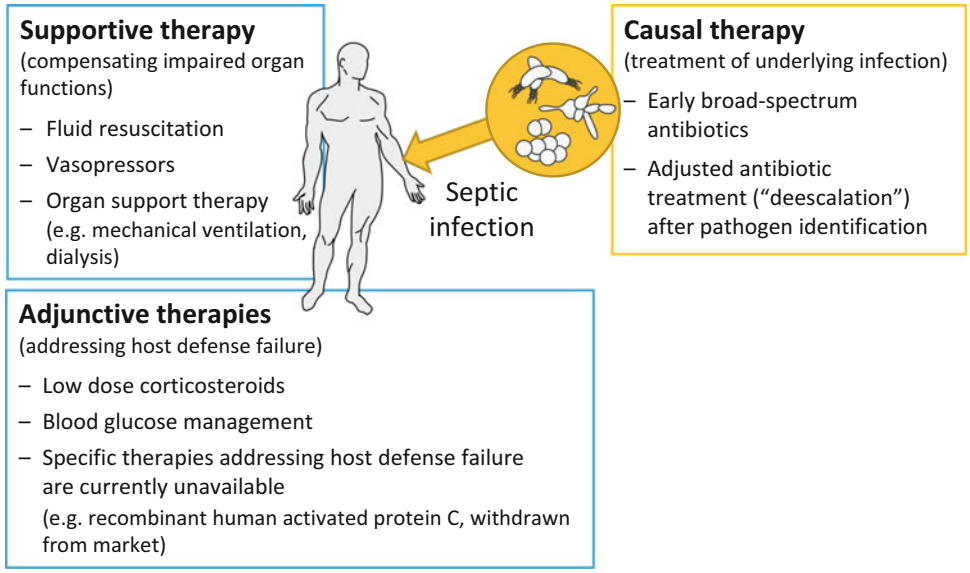
failure. The molecular mechanisms underlying sepsis are, though, not fully understood. However, epidemiological data point toward a rare event in the light of frequent uncomplicated infections receiving antibiotic prescriptions across the population (Singer et al. 2019) (Fig. 1).

Currently, sepsis is best conceptualized as an imbalanced and dysfunctional host response induced by failed control of pathogens at the site of infection. Numerous factors including age, gender, genetic predisposition, and comorbidities influence susceptibility to the disease (Paoli et al. 2018; Taudien et al. 2016). Importantly, damaging processes in the course of sepsis seem to differ depending on tissues, their regenerative capacity, and cell type. For example, sepsis-associated organ dysfunction is often reversible, and necrotic or apoptotic cell death is a rare event in parenchymal organs of septic patients (Hotchkiss et al. 1999). In contrast, immunoparalysis can be attributed to cell death of immune cells, in particular lymphocytes (Hotchkiss et al. 2013).

Despite tremendous research efforts during recent decades, no host-directed *adjunctive therapy* for sepsis exists (Fig. 2). Numerous clinical studies on candidate mediators failed, and only recently, meta-analyses of the clinical trials indicated that certain beneficial effects might have escaped notice due to the highly individual disease courses and etiologies. For example, analysis of data on antibodies neutralizing the pro-inflammatory cytokine anti-TNFα suggests a benefit for a subset of patients (Marshall 2014). Current efforts aim at identification of subgroups of patients that will respond to specific interventions, e.g., immune modulatory therapy or adjunctive therapies (Marshall 2014; Seymour et al. 2019). Besides individualized treatment approaches, targeted drug delivery might hold potential for improved sepsis therapies.

Immune Responses

The occurrence of hyperinflammation and immunoparalysis in sepsis patients raises



Sepsis, Fig. 2 Current options to treat sepsis
Sepsis therapy includes "causal" (anti-infective), "supportive" (replacing impaired organ function), and "adjunctive" (interfering with the failing host response) treatment options

questions concerning the causes of these fatal dysfunctions of the immune system.

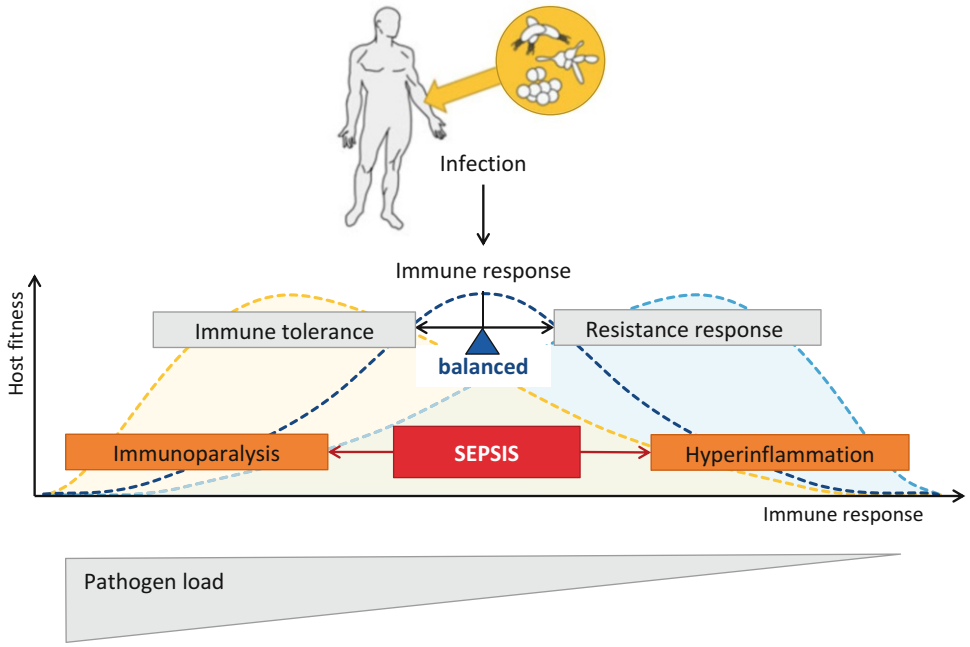
Regularly, higher organisms react to invading microbes with two alternative response patterns. In addition to the well-known resistance responses of the immune system, which result in pathogen clearance, "disease tolerance" has been acknowledged as an alternative adaptive strategy of the host (Medzhitov et al. 2012). Disease tolerance enables the organism to sustain a certain pathogen load without marked damage or disease symptoms (i.e., organ failure). Disease tolerance requires maintenance of cell and organ functions of the infected host by continuous repair reactions despite persistent pathogen burden. Immune resistance and disease tolerance responses enable the resolution of infection and the restoration of health.

In sepsis, the concerted resistance and tolerance responses of the host fail. Exuberant resistance responses cause a hyperinflammatory state, i.e., SIRS, and collateral tissue damage. Dysregulated disease tolerance might result in immunoparalysis, recurrent or chronic infections, and corresponding damage caused by disseminating pathogens (Fig. 3).

Significant progress has been made regarding the understanding of the causes of dysregulated resistance and tolerance and consequently of hyperinflammation and immunoparalysis in sepsis patients. Both response patterns have been attributed to specific traits of the infecting pathogen or the host. However, increasing evidence indicates pathogen dose as key for causing these opposing reactions (Bauer et al. 2018). Innate immune cells challenged with low amounts of pathogens were shown to generate energy-demanding resistance responses, whereas high pathogen load will increasingly perturb cell metabolism. Under these conditions, a shift from resistance reactions toward maintenance and repair activities seems to occur, which ultimately enables tolerance toward pathogens. Induction of resistance and tolerance depending on the pathogen burden might help the cells and tissues to integrate challenges by various microbes and other stressors, in particular in the critically ill, and thus fulfill the basic fitness and survival requirements of cells and organs (Fig. 3).

The Role of Parenchymal Tissues

While the activated immune system during phases of acute infections is highly energy demanding, the overall limited energy supply has to be



Sepsis, Fig. 3 Immune response patterns to infection and development of sepsis
In sepsis, the balanced interaction of immune cells, pathogens, and parenchyma upon local, uncomplicated infections gets out of control. Resistance responses progress to systemic hyperinflammation, whereas excessive tolerance might result in immunoparalysis. Both immune response patterns impact host fitness by causing organ damage. Pathogen load and energy metabolism have been suggested as key mediators of the dichotomous immune responses

redistributed, which might result in parenchymal metabolic shut-down. Indeed, critical illness is frequently associated with reduced oxygen consumption, metabolic adjustments, and decreased organ function. Importantly, this organ dysfunction is not associated with markedly increased cell death and is usually quickly restored after episodes of sepsis (Hotchkiss et al. 1999). This, together with drastically altered metabolism, has led to the concept of "hibernation" of the failing organs (Stanzani et al. 2020). These topics are active research areas aiming at alternative treatment approaches for the attainment of metabolic homeostasis and restoration of organ function during and after sepsis.

Molecular Mechanisms Mediating (Mal-) adaptive Responses to Infection

Molecular signaling events mediating resistance and tolerance responses to infections have been investigated in cellular and animal models. Given the crucial role of a balanced energy budget, the emerging involvement of energy sensing signaling pathways does not come as a surprise. Both in vitro and in vivo data indicate involvement of the mechanistic target of rapamycin (mTOR) signaling pathway in the induction of **resistance** responses to infectious microbes. mTOR is known as a central mediator of anabolic processes. Together with the signaling proteins of the phosphoinositide 3-kinase (PI3K) family, mTOR is controlling energy demanding cell functions, such as proliferation or differentiation, in addition to resistance responses of immune cells (Cheng et al. 2014).

Experimentally, sepsis-induced hyperinflammation could already be effectively treated by drugs targeting mTOR as the key mediator of enduring resistance responses (Yen et al. 2013). In septic mice, the mTOR inhibitor rapamycin significantly suppressed the hyperinflammatory response and decreased mortality.

Signaling reactions that trigger the development of disease **tolerance** are less well understood. Recent investigations revealed AMP-activated protein kinase (AMPK) as a critical mediator of tolerance responses to infections (Bauer et al. 2018). In contrast to mTOR, AMPK activity is induced at low cellular energy levels. In pathogen-challenged cells, the signaling protein mediates maintenance and repair reactions, including autophagy of cellular organelles. A key role of AMPK has been proposed for tissue damage control in parenchymal cells in states of disease tolerance (Soares et al. 2014).

Taken together, resistance and tolerance responses triggered by invading pathogens appear to be part of evolutionary conserved and interdependent reaction patterns of the organism to environmental stress in general (Bauer et al. 2018; Stanzani et al. 2020).

Pharmacological Intervention

Current Standard of Care

Current therapeutic concepts mainly focus on "*causal" therapy*, i.e., early anti-infective therapy, combined with circulatory support by volume resuscitation and additional vasopressors (*"supportive" therapy*), if a mean arterial pressure of 65 mm Hg cannot be achieved by volume resuscitation alone (Rhodes et al. 2017).

Antimicrobial therapy should be initiated as early as possible after diagnosis. Importantly, standard samples for microbiological assessment (including blood cultures) should be taken before starting antibiotics. Initial therapy will, in general, rely on empirical broad-spectrum therapy with one or more antimicrobials (including antifungal or antiviral therapy, if applicable). After identification of the causative pathogen, the antibiotic regimen should be narrowed as appropriate (Rhodes et al. 2017).

According to the Surviving Sepsis Campaign (SSC) guidelines, initial resuscitation from sepsis-induced hypoperfusion should be initiated immediately after sepsis diagnosis using IV crystalloids. If a target mean arterial pressure of 65 mm Hg cannot be achieved by fluid resuscitation alone, norepinephrine is recommended as first-choice vasopressor. Vasopressin or epinephrine can be added to raise the arterial pressure to the target values or to reduce norepinephrine dosage (Rhodes et al. 2017). The SSC as a "one world" initiative has been criticized as primarily suitable for limited income countries, and a more individualized approach regarding hemodynamic monitoring and catecholamine support will be applicable in high-income countries.

The present therapeutic approaches are summarized in Fig. 2.

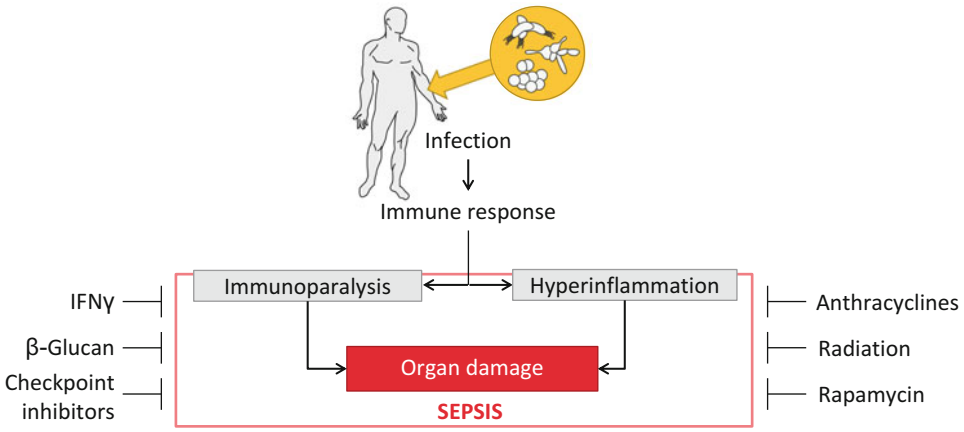
The only currently used immunomodulatory intervention in septic shock is the administration of "stress doses" of hydrocortisone. This approach yielded controversial results (Suffredini 2018) and reflects a prominent example for the need to define subgroups of patients prior to initiation of therapy (personalized medicine, "theranostics").

There is currently no approved therapy that targets the dysbalanced immune response. Moreover, no specific therapies exist to pharmacologically treat organ dysfunction, since recombinant human activated protein C was withdrawn from the market in 2011.

Novel Treatment Approaches – Restoring Immune and Organ Function

From the immunological point of view, the sepsis paradigm currently shifts from a focus on simple "hyperinflammation" to a multifaceted disruption of immune balance, as many patients present with signs of "immunoparalysis." This is best reflected in current trials studying the potential of immunostimulatory therapies, such as growth factors or check point inhibitors as adjunct options in sepsis therapy (Hotchkiss et al. 2013). Leentjens et al. demonstrated partial reversion of immunoparalysis of humans by treatment with IFN-γ in vivo (Leentjens et al. 2012). Immunostimulatory agents used in preclinical models cover a broad spectrum of agents, including pathogenic yeast *Candida albicans* and its cell wall component β-glucan (Novakovic et al. 2016). However, these approaches will have to rely on improved patient stratification.

In line with the critical balance of mTOR and AMPK, hyperinflammation during sepsis can be



Sepsis, Fig. 4 Evolving therapeutic concepts to modulate resistance and tolerance responses in sepsis models Currently studied options to modulate failing immune responses in sepsis. These specific interventions aim to suppress organ damage provoked by either immunopathology or immunoparalysis in the course of sepsis

suppressed by the immunosuppressive and anti-proliferative agent rapamycin (Yen et al. 2013). Additional options to treat hyperinflammatory sepsis have been revealed by Figueiredo et al. (2013). In a groundbreaking study, these authors screened a drug library for candidates that affect inflammatory reactions in a macrophage cell line. Anthracyclines – commonly applied for the therapy of solid cancers – were identified to prevent organ dysfunction in mouse models of polymicrobial sepsis independent of changes in pathogen burden. Apparently, low doses of anthracyclines induce a DNA damage response and autophagy, which are assumed to be the mechanisms underlying tissue protection and anti-inflammatory effects. Interestingly, irradiation produced similar effects. Together, these data indicate that low doses of stressors, such as toxins or irradiation, stimulate cellular repair and thereby induce cross-protection, i.e., generally increased resilience. The anti-inflammatory effects of anthracylines or radiation might be connected to energy demands. Reactions to environmental stressors and inflammatory responses compete for energy sources. The additional energy requirement also affects protein synthesis and cell proliferation. Accordingly, treatment of cells with anthracylines or radiation seems to lower the immune response in favor of tolerance reactions. Morbidity induced by systemic hyper-inflammation decreases, and the organism exhibits a state of "disease tolerance."

Together, these interventions (Fig. 4) targeting immunopathology and immunoparalysis during the course of sepsis might add to the therapeutic armamentarium of personalized care in the field of infection-driven organ dysfunction, which discriminates sepsis from uncomplicated infection.

Cross-References

- AMP-activated Protein Kinase
- Cytokines
- Inflammation
- mTOR Signaling Pathways
- β-Lactam Antibiotics

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Septic Shock

► Sepsis

Serine/Threonine Protein Kinase Phosphorelay Modules

▶ MAP Kinase Cascades

Serotonergic Hallucinogen

▶ Psychedelic Drugs

Serotonin

▶ Serotoninergic System

Serotoninergic System

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Synonyms

5-hydroxytryptamine = 5-HT (= enteramine); Serotonin

Definition

The serotoninergic system is one of the oldest neurotransmitter/hormone systems in evolution, which may explain why 5-HT interacts with such a diversity of receptors of the G protein-coupled family (type A) and the ligand-gated family Cys-loop family, similarly to acetylcholine, GABA, or glutamate. 5-HT was discovered in the gut in the 1930s and called enteramine and then rediscovered in the 1940s in the blood and called serotonin, since it also constricted smooth muscle in the vessels. 5-HT is synthesized from l-tryptophan; there are two tryptophan hydroxylases forming 5-hydroxytrytophan (5-HTP), which by the l-amino acid decarboxylase leads to 5-HT. 5-HT can be conjugated with glucuronide or sulfate. In the nervous system, 5-HT is metabolized via monoamine oxidase to 5-hydroxyindolacetaldehyde and finally to 5-hydroxyindolacetic acid (via aldehyde dehydrogenase). It can also lead to 5-hydroxytryptophol by an aldehyde reductase in some peripheral nerves. Thus, 5-HT acts as a neurotransmitter with all the typical features, such as intracellular storage, activity-dependent release, the existence of both pre- and post-synaptic receptors, and an active uptake system, via the serotonin transporter and metabolizing/inactivating enzymes. 5-HT also acts as a hormone, released into the blood or gut to work more distantly.

Basic Characteristics

Physiology

The main source of 5-HT (95%) is from enterochromaffin cells of the gut, where 5-HT is synthesized from tryptophan. It can be released into the gut lumen, e.g., as a reaction to pressure and act on receptors located on the smooth muscle, or released into the portal blood circulation, by a variety of nervous or alimentary stimuli. 5-HT is also found in enteric neurons. In the blood, the vast majority of 5-HT is in storage granules from platelets, which are endowed with a very active uptake system (they do not synthesize 5-HT). Large amounts of 5-HT are released during platelet aggregation, and it can act locally on endothelial cells and vascular smooth muscle. 5-HT is also present in mast cells. In the central nervous system (CNS) and peripheral nervous system (PNS), 5-HT acts as a neurotransmitter on a variety of

receptors, which may be located pre- or postsynaptically. 5-HT is also found in the pineal gland, where it serves as a precursor for the synthesis of melatonin by 5-HT-*N*-acetyltransferase and hydroxyindole-*O*-methyltransferase, under the control of the clock genes of the suprachiasmatic nucleus, which modulates enzyme activity levels up to 50-fold during the circadian cycle.

Multiple 5-HT Receptor Subtypes

There are at least 15 different 5-HT receptors, and the system may be more complex (see Tables 1, 2, 3 and 4). With the exception of 5-HT_3 receptors (ligand-gated ion channels), 5-HT receptors belong to the G-Protein-Coupled Receptors (GPCR) superfamily, which is one of the most complex families of neurotransmitter receptors. Some 5-HT receptors have multiple splice variants (5-HT_{2C}, 5-HT_4, 5-HT_7) or RNA-edited isoforms (5-HT_{2C}). There is evidence that homo- and hetero-dimerization (e.g., 5-HT_{2C}, 5-$HT_{1B/1D}$, 5-HT_{2C}/GHS-R1a) can occur. We have proposed a classification system based on operational, structural, and transductional criteria (Humphrey et al. 1993; Hoyer et al. 1994; Hartig et al. 1996) which has been progressively refined (Hoyer and Martin 1997; Hannon and Hoyer 2008). The current classification (Barnes et al. 2021) favors an alignment of nomenclature with the human genome to account for species differences. Seven families of 5-HT receptors have been recognized, 5-HT_1 to 5-HT_7. Yearly updates about 5-HT receptors and ligands can be found in the BJP Concise Guide to PHARMACOLOGY (Alexander et al. 2019).

Most 5-HT receptors, except 5-HT_3 which are Cys-loop ligand-gated channels with structural analogy to nicotinic or $GABA_A$ receptors, belong to the GPCR family A. Lowercase denomination means that the receptor is not functionally defined in native in vitro or in vivo systems. The 5-HT_{1C} slot is empty, as it was renamed 5-HT_{2C}. There are no lowercase for 5-HT_3 receptor subunits, as they may form heteromers for which in vivo proof is not yet established, although probable.

The 5-HT_1 receptor class comprises five receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E}, and 5-HT_{1F}) which, in humans, share 40–63% overall sequence identity and couple somewhat preferentially to $G_{i/o}$ to inhibit cAMP formation (see Table 2). 5-ht_{1E} receptors have a lowercase appellation to denote that endogenous receptors with a physiological role have not yet been reported or may not even exist in rodents (rat, mouse). In contrast, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors have been documented functionally. The 5-HT_{1C} designation is vacant, as the receptor was renamed 5-HT_{2C}, due to structural, operational, and transductional similarities with the other two members of the 5-HT_2 receptor family.

5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors exhibit 46–50% overall sequence identity and couple preferentially to $G_{q/11}$ to increase inositol phosphates and cytosolic [Ca^{2+}] (see Table 3).

5-HT_3 receptors belong to the ligand-gated Cys-loop ion channel receptor superfamily, similar to the nicotinic acetylcholine or $GABA_A$ receptors, and share electrophysiological and structural patterns. The receptors are found on central and peripheral neurons where they trigger rapid depolarization due to the opening of non-selective cation channels (Na^+, Ca^{2+} influx, K^+ efflux). The response desensitizes and resensitizes rapidly. The native 5-HT_3 receptor, as revealed by electron microscopy, is a pentamer. There are five potential different subunits, 5-HT_{3A}, 5-HT_{3B},

Serotoninergic System, Table 1 5-HT receptor families and receptor subtypes

5-HT_1	5-HT_2	5-HT_3	5-HT_4	5-ht_5	5-HT_6	5-HT_7
5-HT_{1A}	5-HT_{2A}	5-HT_{3A}	5-HT_4	5-ht_{5A}	5-HT_6	5-HT_7
5-HT_{1B}	5-HT_{2B}	5-HT_{3B}		5-ht_{5B}		
5-HT_{1D}	5-HT_{2C}	5-HT_{3C}				
5-ht_{1E}		5-HT_{3D}				
5-HT_{1F}		5-HT_{3E}				

Serotoninergic System, Table 2 5-HT_1 receptor nomenclature as proposed by the NC-IUPHAR Subcommittee on 5-HT receptors

Nomenclature	5-HT_{1A}	\$5-HT_{1B}	5-HT_{1D}	5-ht_{1E}	5-HT_{1F}
Previous names	–	5-$HT1_{D\beta}$	5-$HT1_{D\alpha}$	–	5-$ht_{1E\beta}$, 5-HT_6
Selective agonists	8-OH-DPAT	Sumatriptan	Sumatriptan	–	LY 334370
	(R)-UH301	Eletriptan	Eletriptan		LY 334864
	F13714	L 694247	PNU 109291		
	Befiradol	CP93129(rodent)	L775606		
Selective antagonists (pKB)	WAY100635 (8.7)	GR 55562 (7.4)	BRL 15572 (7.9)	–	–
	Robalzotan (9.2)	SB 224289 (8.5)			
	NAN190 (9.4)	SB 236057 (8.9)			
Radioligands	[^{3}H] WAY100635	[^{125}I]GTI	[^{125}I]GTI	[^{3}H]5-HT	[^{125}I]LSD
	[^{3}H]8-OH-DPAT	[^{125}I]CYP (rodent)	[^{3}H] Sumatriptan		[^{3}H]LY 334370
	[^{3}H] Robalzotan	[^{3}H]Sumatriptan	[^{3}H]GR 125743		
	[^{3}H]pMPPF	[^{3}H]GR 125743			
G protein effector	Gi/o	Gi/o	Gi/o	Gi/o	Gi/o
Gene/chromosomal localization	HTR1A/ 5q11.2-q13	HTR1B/6q13	HTR1D/ 1p34.3-36.3	HTR1E/ 6q14-15	HTR1F/3p11-p14.1
Structural information	h421 P8908	h390 P28222	h377 P28221	h365 P28566	h366 P30939
	m421 Q64264	m386 P28334	m374 Q61224		m366 Q02284
	r422 P19327	r386 P28564	r374 P28565		r366 P30940

5-HT_{3C}, 5-HT_{3D}, and 5-HT_{3E}. Currently, the consensus is that native receptors are best reproduced by the heteromeric combination of 5-HT_{3A} and 5-HT_{3B} subunits. 5-HT_{3B} homomers lack functions (as is probably the case for either of 5-$HT_{3C,3D,3E}$). 5-HT_{3A} subunits can form homopentamers, but to become really functional, they have to form heteropentamers (with, e.g., 5-HT_{3B}) with two 5-HT_{3A} subunits. It has been suggested that 5-HT_3 receptors can heterodimerize with other members of the superfamily (e.g., nicotinic alpha$_7$), but this has not been demonstrated in situ.

5-HT_4, 5-HT_6, and 5-HT_7 receptors all couple preferentially to G_s and promote cAMP formation, yet they are regarded as distinct receptor classes due to their limited (<35%) overall sequence identities. This subdivision is arbitrary and may be subject to future modification.

Two subtypes of the 5-ht_5 receptor (5-ht_{5A} and 5-ht_{5B}), sharing 70% overall sequence identity, have been found in rodents. The human 5-ht_{5B} receptor gene does not encode a functional protein, due to the presence of stop codons in its coding sequence. Human recombinant 5-ht_{5A} receptors inhibit forskolin-stimulated cAMP production, although the receptor may also couple positively to cAMP production. Currently, a physiological readout for this receptor is still missing, although A-843277, a selective 5-ht_{5A} antagonist, has antidepressant/antipsychotic properties in rodent models.

Additional 5-HT receptors have been defined phenotypically and pharmacologically, although a corresponding gene product encoding the receptor has not yet been identified. As long as their structure is unknown, these receptors are regarded as orphans. However the so-called "5-HT_1-like" receptor mediating direct vasorelaxation actually corresponds to the 5-HT_7 receptor. The situation with the remaining orphan receptors (see Hoyer

Serotoninergic System, Table 3 5-$HT_{2, 3, 4}$ receptor nomenclature as proposed by the NC-IUPHAR Subcommittee on 5-HT receptors

Nomenclature	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}[b]	5-HT_3	5-HT_4
Previous names	D/5-HT_2	5-HT_{2F}	5-HT_{1C}	M	–
Selective agonists	α-Methyl-5-HT	BW 723C86	Ro 600175	SR 57227	BIMU 8
	DOI[a]	Ro 600175	DOI	m-chlorophenyl-biguanide	RS 67506
	DOB	DOI			ML 10302
Selective antagonists (pKB)	Ketanserin (8.5–9.5)	LY266097 (9.7)	Mesulergine (9.1)	Granisetron (10)	GR 113808 (9–9.5)
	Volinanserin (9.4)	RS127445 (9.5)	SB 242084 (9.0)	Ondansetron (8–10)	SB 204070 (10.8)
	Pimavanserin (9.4)	SB 204741 (7.8)	RS 102221 (8.4)	Tropisetron (10–11)	RS 100235 (11.2)
Radioligands	[^{125}I]DOI	[^{3}H]5-HT	[^{125}I]LSD	[^{3}H](S)-zacopride	[125I]SB 207710
	[^{3}H]DOB		[^{3}H] Mesulergine	[^{3}H]tropisetron	[3H]GR 113808
	[^{3}H] Ketanserin			[^{3}H]granisetron	[3H]RS 57639
	[^{3}H]MDL 100907			[^{3}H]GR 65630	
				[^{3}H]LY 278584	
G protein effector	Gq/11	Gq/11	Gq/11	[c]	Gs
Gene/chromosomal localization	HTR2A/ 13q14-q21	HTR2B/ 2q36.3-q37.1	HTR2C/ Xq24	HTR3/11q23.1-q23.2	HTR4/5q31-33
Structural information	h471 P28223	h481 P41595	h458 P28335	Multi-subunit[d]	h387 Y09756AS
	m471 P35362	m504 Q02152	m459 P34968	5-HT_{3A}, 5-HT_{3B},	m387 Y09587AS
	r471 P14842	r479 P30994	r460 P08909	5-$HT_{3C,}$ 5-HT_{3D},	r387 U20906AS
				5-$HT_{3E.}$	

[a]Also activates the 5-HT_{2C} receptor. [b]Multiple isoforms of the 5-HT_{2C} receptor are produced by RNA editing. [c]The 5-HT_3 receptor is a ligand-gated cation channel that exists as a pentamer of 4TM subunits. [d]Human, rat, mouse, guinea pig, and ferret homologues of the 5-HT_{3A} receptor have been cloned that exhibit species variation in pharmacological profile. A second 5-HT_3 receptor subunit, 5-HT_{3B}, imparts distinctive biophysical properties upon hetero-oligomeric (5-HT_{3A}/5-HT_{3B}) versus homo-oligomeric (5-HT_{3A}) recombinant receptors. The function of the 5-$HT_{3C,3D,3E}$ subunits and other putative members of the family requires further investigations

et al. 1994; Barnes et al. 2021) has not evolved further, and thus the status quo ante remains. In particular, no progress has been made with the "5-HT_{1P} receptor," which is described in the gut and whose pharmacology is reminiscent of the 5-HT_4 receptor, with the restriction that some of the ligands described as 5-HT_{1P} selective, like the 5-HT dipeptides, do not affect 5-HT_4 receptors. The 5-HT_{1P} receptor could be a heterodimer, hence the separate pharmacological profile; however the putative partners remain to be identified.

Pathophysiology/Clinical Applications

5-HT has been implicated in the etiology of numerous disease states, including depression, anxiety, social phobia, schizophrenia, Parkinson's disease and other movement disorders, obsessive compulsive disorders, aggression, panic and sleep disorders, migraine, vascular and pulmonary hypertension, eating disorders, addiction, nausea/vomiting, irritable bowel syndrome (IBS), and other functional GI disorders, by interacting at different receptors or transporters (see 6).

Serotoninergic System, Table 4 5-$HT_{5, 6, 7}$ receptor nomenclature as proposed by the NC-IUPHAR Subcommittee on 5-HT receptors

Nomenclature	5-ht_{5A}	5-ht_{5B}	5-HT_6	5-HT_7
Previous names	5-$HT_{5\alpha}$	–	–	5-HT_X, 5-HT_1-like
Selective agonists	–	–	–	–
Selective antagonists (pK_B)	SB 699551 (8.0)	–	Ro 630563 (7.9)	SB 258719 (7.9)
			SB 271046 (7.8)	SB 269970 (9.0)
			SB 357134 (8.5)	
Radioligands	[^{125}I]LSD	[^{125}I]LSD	[^{125}I]SB 258585	[^{125}I]LSD
	[^{3}H]5-CT	[^{3}H]5-CT	[^{125}I]LSD	[^{3}H]SB 269970
			[^{3}H]5-HT	[^{3}H]5-CT
				[^{3}H]5-HT
G protein effector	$G_{i/o}$	None identified	G_s	G_s
Gene/chromosomal localization	HTR5A/7q36.1	htr5b/2q11-q13	HTR6/1p35-36	HTR7/10q23.3-24.3
Structural information	h357 P47898	m370 P31387	h440 P50406	h445 P34969^{AS}
	m357 P30966	r370 P35365	m440 NP_067333	m448 P32304
	r357 P35364		r438 P31388	r448 P32305^{AS}

Indeed, 5-HT is also a substrate for the 5-HT transporter, itself an important target in the treatment of depression and the extended range of anxiety disorder spectrum (GAD, OCD, social and other phobias, panic and post-traumatic stress disorders). The 5-HT transporter is the target for SSRIs (selective serotonin reuptake inhibitors) such as fluoxetine, paroxetine, fluvoxamine, and citalopram or the more recent dual reuptake inhibitors (for 5-HT and noradrenaline, also known as SNRIs) such as venlafaxine. There are still efforts to develop triple uptake inhibitors (5-HT, NE, and DA) and/or drugs combining receptor modulation and uptake blockade.

5-HT_{1A} receptor agonists, such as buspirone, tandospirone, or gepirone, were developed for the treatment of anxiety and depression. Furthermore, the 5-HT_{1A} receptor and β-adrenoceptor antagonist, pindolol, was reported to enhance the therapeutic efficacy and shorten the onset of action of SSRIs upon co-administration in severely depressed patients. It is suggested that the combination therapy functions provided that sufficient 5-HT_{1A} receptor occupancy is reached. The antidepressants vilazodone and vortioxetine combine 5-HT uptake inhibition and 5-HT_{1A} agonism. A number of antipsychotics such as clozapine, ziprasidone, quetiapine, aripiprazole, lurasidone, and cariprazine also share 5-HT_{1A} agonism, which may help with hypofrontality and memory impairment.

5-HT_{1B} receptors have both a CNS and a vascular distribution and are the targets of the antimigraine drug sumatriptan, a non-selective 5-$HT_{1D/1B}$ receptor agonist, ergolines, and many other triptans (ergotamine, dihydroergotamine (DHE), zolmitriptan, naratriptan, rizatriptan, eletriptan, almotriptan, donitriptan, etc.).

However, most of the triptans have at least equal affinity to 5-HT_{1D} receptors, which are not expressed in vessels. The 5-HT_{1D} approach to target migraine with 5-HT_{1D} agonists, as a non-vascular target, did not succeed. Indeed, the selective 5-HT_{1D} receptor agonist PNU-142633 displayed no efficacy in migraine patients.

There is no evidence for any clinical relevance of the 5-ht_{1E} receptor. By contrast, since sumatriptan and naratriptan also bind to the 5-HT_{1F} receptor, it has been hypothesized that 5-HT_{1F} might be a target for antimigraine drugs. 5-HT_{1F} receptor mRNA is present in the trigeminal ganglia, stimulation of which leads to plasma extravasation in the dura, a component of neurogenic inflammation linked to migraine. Lasmiditan

(COL-144, LY573144), a selective 5-HT_{1F} receptor agonist, has been developed and registered for the treatment of migraine, with no evidence of cardiovascular side effects.

Ketanserin, a selective 5-HT_{2A} antagonist was initially developed to treat hypertension, but 5-HT_{2A} receptor antagonism as antihypertensive principle has failed. LSD and other psychedelics (e.g., psilocybin, MDMA, and N,N DMT) most probably produce their effects by activating central 5-HT_{2A} receptors, although their selectivity vis- à -vis 5-HT_{2B} and 5-HT_{2C} receptors is limited. Psilocybin and MDMA received FDA breakthrough therapy status for the treatment of treatment-resistant depression and PTSD, respectively. There is a renewed interest in LSD and other psychedelics for the treatment of a number of neuropsychiatric conditions, including substance use disorders. 5-HT_{2A} receptor antagonists such as risperidone, ritanserin, seroquel, olanzapine, volinanserin (MDL 100907), or cariprazine have been developed for the treatment of schizophrenia. The combination of dopamine D_2 and 5-HT_{2A} receptor antagonism may still explain the beneficial antipsychotic activity of drugs such as clozapine, olanzapine, seroquel, and many others. More recently, pimavanserin was approved by the US FDA to treat hallucinations and delusions associated with Parkinson's disease, although it did not meet clinical endpoints in schizophrenia.

BW 723C86 is a selective agonist for the rat 5-HT_{2B} receptor, less selective at human receptors, and was investigated in various neuropsychiatric indications. However, 5-HT_{2B} receptor activation is responsible for the valvulopathies reported for appetite suppressant preparations containing fenfluramine, dex-fenfluramine, or benfluorex; therefore 5-HT_{2B} agonism is strictly banned from all drug development programs. 5-HT_{2B} antagonists have been in clinical trials with indications such as migraine, pulmonary hypertension, irritable bowel syndrome, and various forms of fibrosis, but no compound has been registered so far.

The anxiogenic component of mCPP may be mediated by 5-HT_{2C} receptor activation, and selective 5-HT_{2C} receptor antagonists such as agomelatine display anxiolytic/antidepressant properties in animal models. Central 5-HT_{2C} receptor activation produces a tonic, inhibitory influence upon frontocortical dopaminergic and adrenergic, but not serotonergic transmission. Agomelatine a 5-HT_{2C} antagonist, combined with melatonin 1/2 receptor agonist, is a clinically effective antidepressant. Lorcaserin, a 5-HT_{2C} receptor agonist, was registered to treat obesity and eating disorders but was withdrawn (Tox issues). 5-HT_{2C} agonists are being investigated in obesity, substance use disorders, depression, and schizophrenia. The 5-HT_{2C} agonist vabicaserin entered phase II trials in schizophrenia, but the clinical outcomes were moderate, and development stopped.

5-HT_3 receptor antagonists, e.g., ondansetron, granisetron, and tropisetron, are used clinically to treat chemotherapy- and radiotherapy-induced nausea and vomiting. Since 5-HT_3 receptor activation in the brain leads to dopamine release, and 5-HT_3 receptor antagonists produce central effects similar to those of antipsychotics and anxiolytics, schizophrenia and anxiety were also considered as potential indications. 5-HT_3 receptor antagonists have been reported to have cognition enhancing effects and active in pain and migraine models, but neither of these did materialize in clinical studies. On the other hand, alosetron is now indicated for women suffering from IBS with diarrhea (IBS-D), although under very strict conditions (black box).

Selective 5-HT_4 receptor ligands have therapeutic utility in a number of disorders, including cardiac arrhythmia, neuro-degenerative diseases, depression, urinary incontinence, and GI motility disorders. Cisapride, a gastroprokinetic agent, acts as an agonist at the 5-HT_4 receptor but was withdrawn due to side effects (QT prolongation). Metoclopramide is a 5-HT_4 agonist, with dopaminergic activity. Tegaserod, a 5-HT_4 receptor partial agonist, was used to treat constipation-predominant IBS (IBS-C) and constipation and, after a withdrawal, has been reintroduced under a limited schedule. The 5-HT_4 agonist prucalopride increases bowel movement in volunteers and is registered to treat chronic constipation. Finally, the 5-HT_4 antagonist piboserod is used to treat atrial fibrillation.

Multiple antipsychotics (clozapine, olanzapine, fluperlapine, and seroquel) and

antidepressants (clomipramine, amitriptyline, doxepin, and nortriptyline) are 5-HT_6 receptor antagonists. This attribute suggested an involvement of the 5-HT_6 receptor in psychiatric disorders. Selective 5-HT_6 receptor antagonists have positive effects in preclinical models of memory impairment and cognition. The 5-HT_6 receptor antagonist idalopirdine shows positive clinical effects in Alzheimer's disease when administered in combination with the cholinesterase inhibitor donepezil.

The 5-HT_7 receptor has affinity for multiple atypical antipsychotics, e.g., clozapine, risperidone, amisulpride, aripiprazole, or lurasidone, and various antidepressants. The presence of 5-HT_7 sites in the limbic system and thalamocortical regions suggests a role in affective disorders, whereas the expression in the supra chiasmatic nucleus supports a role in circadian rhythms. Various 5-HT_7 antagonists were/are investigated in depression, psychosis, schizophrenia, and circadian disorders.

Cross-References

► Antidepressant Drugs

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Sertoli Cell Barrier

► Blood-Testis Barrier

Severe Eosinophilic Asthma

► Interleukin-5

Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor

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Definition

Sex steroid receptors are members of the steroid hormone receptor (SHR) family that ligand-

dependently regulate functions of the sexual organs. Sex steroid receptors are the androgen receptor (AR), the estrogen receptor α and β (ERα, ERβ), and the progesterone receptor (PR) (OMIM 313700, 2019; OMIM 133430, 2020; OMIM 601663, 2020; OMIM 607311, 2019; respectively).

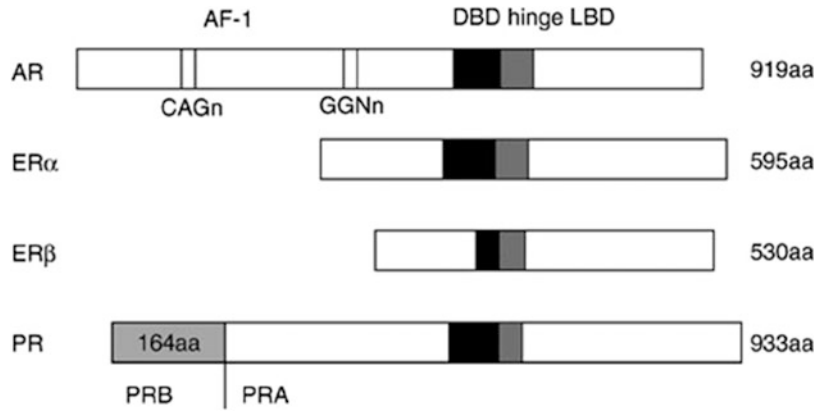
Basic Characteristics

The subgroup of SHRs belongs to the superfamily of nuclear receptors, which transactivate target genes ligand-dependently. Unliganded SHRs are associated with large multiprotein complexes of chaperones in the cytoplasm, in contrast to other nuclear receptors. SHRs comprise the glucocorticoid receptor and the mineralocorticoid receptor and the sex steroid receptors.

SHRs are built in a modular structure with similar structure elements. They contain a DNA-binding domain (DBD), a hinge region with a nuclear location signal (NLS), a ligand-binding domain (LBD), and several transcriptional activation functions (Fig. 1). Their ligands are fat-soluble steroid hormones derived from cholesterol that bind to the LBD of their specific intracellular SHR after diffusing into the cell. After binding of the steroid hormone (K_d between 0.1 and 4 nM), the conformation of the SHR changes, exposing the NLS and the complex of steroid hormone, and SHR gains access to the nucleus (Fig. 2). Utilizing the two zinc fingers of their DBD, SHRs bind as homodimers to unique DNA sequences called hormone response elements (HREs). The HRE is comprised of two half-sites organized as palindrome with a three nucleotide spacer. SHRs regulate the expression of target genes after association with large multisubunit complexes that contain transcriptional co-activators such as histone acetylases and several other proteins that facilitate transcription. Several signaling pathways furthermore influence the activity of SHRs, by modifying either SHRs directly or partner proteins. SHRs can also act without binding to DNA via interaction with other transcription factors, thereby altering their own or their partner's properties.

During the last years, there is increasing evidence that SHR is also associated with the plasma membrane. Rapid, membrane-initiated steroid signals (MISS) were characterized in cell and mouse models for all SHR. Selective pharmacological tools are on the way to dissect the role of genomic and non-genomic signals in vivo.

The physiological and pathophysiological roles of the sex steroid receptors are diverse and will be summarized separately for AR, ERα, ERβs, and PR in the following paragraphs. Estrogen-related receptors (ERRs) comprising ERR-alpha, ERR-beta, and ERR-gamma (gene symbol ESRRA, ESRRB, ESRRG) share structural and functional similarities with ERs. They are orphan receptors indicating that there is no known natural ligand and are therefore not grouped as SHR.



Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor, Fig. 1 Schematic structures of AR, ERα, ERβ, and PR

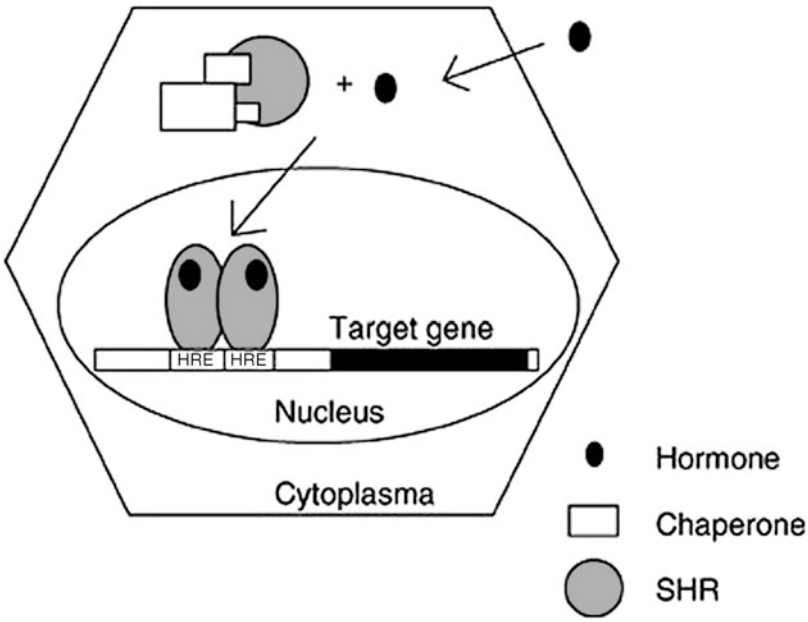
Androgen Receptor

Androgens act via the AR and play an important role in the development and differentiation of the male sexual organ. Furthermore, they are involved in several diseases, the most important being partial and complete androgen insensitivity syndrome (AIS; formerly known as the testicular feminization syndrome), spinal and bulbar muscle atrophy (SBMA; Kennedy's disease), and the neoplastic transformation of the prostate. The two natural occurring androgens are testosterone (T) and the more potent 5α-dihydrotestosterone (DHT). T is mainly produced by the Leydig cells of the testis and can also be produced in most peripheral tissues from the adrenal-produced inactive steroid precursors dehydroepiandrosterone, its sulfate, and androstenedione. T is converted into DHT by the 5α-reductase enzyme expressed in the urogenital tract. Besides positive regulation of target genes by the androgen-loaded AR, there is growing evidence of additional regulation pathways and indirect regulation mechanism of the AR. The expression of specific transcriptional coactivators of the AR in different tissues can fine-tune the transcriptional AR activity. Ligand-independent activation of the AR by protein kinase pathways can circumvent the need for androgens. Non-genomic AR signaling activates c-Src, a tyrosine kinase known to drive prostate metastasis, and p85α the regulatory subunit of PI3K (phosphatidylinositol 3-kinase). In addition, protein-protein interactions of the AR with other transcription factors regulate the transcriptional activity of these partner proteins.

Eight exons of the AR gene encode a protein of around 917 aa depending on two polymorphic regions of polyglutamines (CAG) and polyglycines (GGN) in the N-terminal activation domain. Two isoforms are detected in tissues: the predominant (80%) 110 kD (B isoform) and 87 kD (A isoform). It is under investigation whether the two isoforms also serve different functions.

The structure of the AR comprises an N-terminal transactivation domain of around 500 aa, a DBD of 66–68 aa, and a LBD of 250 aa. The hinge region contains the lysine-rich NLS. The AR possesses two activation functions (AFs): AF-1 in the N-terminal region and the AF-2 core domain in the LBD.

The AF-1 contains two polymorphic regions of CAG and GGN repeats. Normally the number of the 5′ CAG repeats is 11–31 (average 21), whereas up to 50 repeats are found in individuals affected with SBMA. Since the number of glutamines inversely correlates with the transcriptional



Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor, Fig. 2 The classical activation pathway of SHR. SHRs (gray circle) are associated with chaperones (rectangles). After binding of steroid hormones (black circle), SHRs activate target genes in the nucleus. Additional regulation mechanisms, e.g., phosphorylation, are described in the text. For non-genomic signaling, see Arnal (2017)

activity of the AR, these amplified repeats lead to a reduced activity of the AR. The increased size of a polymorphic tandem CAG repeat is associated with the X-linked spinal and bulbar muscular atrophy and may also be associated with oligospermic infertility in men and with low serum androgens in a subset of anovulatory female patients. On the other hand, a shorter CAG length correlates with a higher risk of more severe and earlier onset of prostate cancer probably resulting from a higher activity of the AR. Experimental evidence also correlates increased or prolonged induction of AR activation with a higher incidence or acceleration of prostate cancer.

In the beginning, prostate cancer cells are largely dependent on androgens for growth and survival. Observations that castration is beneficial in prostate cancer made androgen ablation and antiandrogen therapy a standard treatment for patients with metastatic prostate cancer following surgery of the tumor tissue. Antiandrogen therapy includes inhibition of androgen synthesis by aminoglutethimide or ketoconazole and inhibition of 5α-reductase by finasteride in combination with AR antagonists such as flutamide or cyproterone acetate. Second-generation antiandrogens are currently in clinical trials.

Unfortunately, remaining prostate cancer cells eventually adapt to grow in the low-androgen environment, rendering the tumor growth independent of androgens. Androgen-independent prostate cancer may result from one or more of the following mechanism: increased gene copy number; altered interaction of the AR with coregulatory proteins, e.g., resulting from AR mutations, bypassing of the AR pathway; or ligand-independent activation of AR, e.g., by protein kinase pathways and vice versa.

Estrogen Receptor

Estrogens mainly affect the growth and maturation of the female reproductive system and the maintenance of its reproductive capacity [5]. In addition, estrogens act on several other tissues, e.g., on lipid and bone metabolism. Uterus, placenta, and testis are the principal sites of 17-β-estradiol (E2) production. Agonists of ERs are used for treatment of menopausal symptoms, hormone replacement therapy (HRT), osteoporosis, and cardiovascular diseases, whereas antagonists of ERs such as tamoxifen are used for treatment of breast cancer. Pure antagonists of ER that are as effective as tamoxifen without having tamoxifen's partial agonistic effects on ERs are in different phases of testing. The pure antagonist fulvestrant (ICI 183,780) is licensed as treatment for advanced breast cancer. The main isoforms of the human ERs are ERα and ERβ, which display distinct expression patterns. Additional ER isoforms, generated by alternative mRNA splicing, have been identified in several tissues. A cell-specific localization for each of the ER subtypes is found in the majority of the reproductive organs studied. The role of the different ER isoforms in modulating the estrogen response or in tumorigenesis is not completely understood. In addition, ERRs which were coexpressed with ERs can influence the expression level of ER target genes either directly or by interaction with ERs. ERs bind most ligands with similar affinities and display equal transcriptional activation. However, in some assays ER isoforms respond differently to ligands. The naturally occurring phytoestrogen genistein or antiestrogens, such as tamoxifen or raloxifene, are examples of these selective ER modulators (SERMs). The characterization of SERMs that specifically regulate defined functions promises to increase efficacy and reduce side effects in estrogen-regulated processes (Table 1).

ERα

ERα (also called ESR1 and ESRA) is involved in the differentiation and maintenance of reproductive, neural, skeletal, and cardiovascular tissues. Two separate AFs mediate transcriptional activation, the ligand-dependent AF-2 in the LBD and the ligand-independent AF-1 in the N-terminus. After binding of estrogen to the LBD, ERα activates target genes such as the progesterone receptor gene by binding to the estrogen response elements (EREs). Besides this classical activation, nonestrogen-dependent activation of ERα has been described. Signaling pathways and extracellular signals such as EGF, IGF-I, or insulin

Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor, Table 1 Basic characteristics of sex steroid receptors

SHR	Gene map locus	cDNA size	Natural agonist	Binding half-site	Main expression
AR	Xq11–q12	Ca. 919 aa (see text)	5α-dihydrotestosterone (DHT), testosterone (T)	AGAACA	Prostate, male urogenital system, muscle
ERα	6q25.1	595 aa	17β-estradiol (E2)	AGGTCA	Ovary, uterus, mammary gland, vagina, testis (Leydig cells), bone
ERβ	14q22–q24	530 aa, 583 aa, further isoforms	17β-estradiol (E2)	AGGTCA	Ovary, testis (Sertoli and Leydig cells, efferent ducts), prostate, bone, thymus, spleen, brain
PR	11q22	Two isoforms:	Progesterone	AGAACA	Uterus, ovary, central nervous system
		PRA 769 aa			
		PRB 933 aa			

SHR Sex Steroid receptors, *AR* Androgen receptor, *ER* Estrogen receptors, *PR* Progesterone receptor

stimulate phosphorylation of the receptor. Phosphorylation of ERα affects all steps of transcriptional activation, such as ligand binding, dimerization, DNA binding, and interaction with cofactors. Heregulin (NRG1) also called Neuregulin 1 is an example of an extracellular signal modulating ER activity. After binding of NRG1 to its receptor ErbB2 (HER-2), ER is rapidly phosphorylated, followed by transcription of the *PR* gene. Since heregulin promotes hormone-independent growth of breast cancer cells, activation of ER by ErbB2 signaling may be involved in the development of E2-independent cancer cell growth.

Besides the classical activation model, ERα is also found associated with the plasma membrane via palmitoylation, from where it initiates rapid, non-nuclear signaling. These nongenomic, rapid, membrane-initiated steroid signals (MISS) are particularly relevant for endothelial effects of estrogen. MISS-selective agents such as estrogen-dendrimer conjugates and mouse models, e.g., carrying mutations for the palmitoylation site of ERα, will help to dissect genomic and membrane-initiated signals of ERα and their jointly actions.

In addition to the major 66 kDa full-length ERα (ESR66), a 46-kD isoform exists that lacks the 173 N-terminal amino acids which results either from alternative splicing, proteolysis, or translational variation. ESR46 can competitively inhibit the ligand-independent transactivation function of the larger receptor and modulate membrane-initiated estrogen actions. The regulation of its expression that is detected in various cell types and a majority of breast tumors is currently not understood.

ERα can also regulate gene expression by interacting with different transcription factors. For example, interaction of ERα with the c-Rel subunit of NF-κB prevents binding to NF-κB response element resulting in reduced interleukin-6 transcription. Here, ERα acts E2-dependently but without directly binding to DNA. However, the complex formation of ER with the transcription factor Sp1 is hormone-independent and enhances Sp1 binding to DNA. ERα as well as ERβ thereby enhances transcription of the retinoic acid receptor *α1* gene. Other partner proteins are fos/jun family members, which regulate gene expression via AP-1 sites. In this situation E2 can either act as agonist in the presence of ERα or as antagonist in the presence of ERβ. Another possibility to modulate ER signaling is the aryl hydrocarbon receptor (AhR). Ligands for AhR mediate antiestrogenic effects by several pathways.

The measurement of ER has become a standard assay in the clinical management of breast cancer. The presence of ERα identifies those breast cancer

patients with a lower risk of relapse and better clinical outcome. Receptor status also provides a guideline for those tumors that may be responsive to hormonal intervention. But only about half of ER-positive patients respond to hormonal therapies. Of those who respond initially, most will eventually develop an estrogen unresponsive disease following a period of treatment even though ERα is often still present. Mutant receptors and constitutively active receptors as well as hormone-independent activation of the ERα are discussed. The involvement of ERβ isoforms is under investigation.

Analysis of ERα-deficient mice showed that both sexes are infertile and display a variety of phenotypic changes associated with the gonads, mammary glands, reproductive tracts, and skeletal tissues. In addition, both hyperplasia and hypertrophy of adipocytes were found in these mice.

In females, ovariectomy or menopause leads to rapid loss of trabecula bone and osteoporosis indicating that E2 maintains bone mass. E2 enhances bone formation by osteoblasts and suppresses bone resorption by osteoclasts by regulating several important growth factors. In mice, ERα seems to induce growth but not maintenance of trabecular bone, whereas ERβ terminates growth during late puberty. However, while in humans the role of ERα and ERβ in bone is not completely clear, ESRA polymorphism is related to bone density and height during late puberty and at attainment of peak bone density in young men.

ERβ

In 1995 the discovery of ERβ (also called ESR2 and ESRB) explained many actions of estrogens in tissues where no ERα was found. ERβ shows high homology to ERα in the DBD and LBD but encodes a distinct transcriptional AF-1 domain. At least five isoforms, designated ERβ-1 through ERβ-5, are described that differ in their C-terminal sequences and tissue expression patterns or have extended N-termini. This new complexity of isoforms is further enhanced by the fact that ERβ isoforms cannot only heterodimerize with each other but also with ERα. The functional consequences for the action of estrogens depending on the expression pattern are only beginning to be evaluated.

ERβ is highly expressed in the ovary, male organs, and parts of the central nervous system (CNS), but also in other organs such as spleen and thymus. The phytoestrogen genistein binds better to ERβ than to ERα, whereas the partial ERα agonists tamoxifen, raloxifene, and ICI-164384 are antagonists for ERβ. It has been postulated that co-factor recruitment is different for the ERs; however knowledge about this interesting field of selective ER regulation is only beginning to accumulate. Since only ERβ is expressed widely in the male urogenital tract of several animals, it is now under evaluation whether the pronounced effects of E2 in men are caused by direct action of E2 on ERβ in these reproductive organs. The view that E2 acts only indirectly by reducing androgen levels via the CNS clearly has to be corrected.

Analysis of ESRB $^{-/-}$ mice showed fewer and smaller litters than wild-type mice as well as abnormal vascular function and hypertension. The reduction in fertility was attributed to reduced ovarian efficiency. Mutant females had normal breast development and lactated normally. Older mutant males displayed signs of prostate and bladder hyperplasia. *Esr2*-deficient mice furthermore display diverse regulatory defects in the function of the brain, lung, and white blood cells. The results indicated that ESRB is essential for normal ovulation efficiency but is not essential for lactation, female or male sexual differentiation, or fertility.

Progesterone Receptor

The PR is involved in diverse functions in female reproduction, such as implantation of the embryo, and in the maintenance of pregnancy. Progesterone is mainly produced in the corpus luteum in the second half of the menstrual cycle and in early pregnancy, later in the placenta. The PR is expressed in the uterus, ovary, and the CNS. In men there is no known function. Estrogens induce expression of the *PR* gene. PR agonists such as medroxyprogesterone or the synthetic R5020 are called progestins or gestagens.

The human PR exists as two functionally distinct isoforms PRA and PRB transcribed from two promoters from a single gene. PRA lacks the N-terminal 164 aa and is a 769 aa protein. PRB functions as a transcriptional activator in most cell and promoter contexts. In contrast, PRA is transcriptionally inactive and functions as a strong ligand-dependent transdominant repressor of SHR transcriptional activity. Different cofactor interactions were demonstrated for PRA and PRB, probably due to an inhibitory domain within the first 140 aa of PRA, which is masked in PRB. Both PR isoforms, however, repress estradiol-induced ER activity when liganded. Several other mRNA isoforms are present in PR-positive tissues such as breast cancer with unknown clinical significance.

In addition to the classical activation pathway, a substantial part of PR action is mediated by non-genomic signaling by interaction of PR with other signaling pathways. For example, rapid, non-genomic cytoplasmic signaling by PR is initiated by binding to c-Src via a specific polyproline motif in the N-terminal domain of PR, leading to downstream MAP kinase pathway activation.

Mice models reveal that both PR forms are physiologically important. Mice lacking the *PR* gene fail to ovulate, are infertile, and have impaired thymic function. Selective PRA-deficient female mice are infertile due to reduced oocyte and uterine deficiency in implantation. However, these mice had normal mammary epithelium proliferation and differentiation and showed normal thymic involution. In mice, PR regulates expression of proteases that degrade the follicular wall, thereby facilitating ovulation.

In breast cancer patients, total PR status is measured for hormonal treatment. The presence of PR is associated with increased survival rates and hormonal responsiveness of mammary tumors. PR agonists are widely used in contraception, hormone replacement therapy (HRT), breast cancer, and endometrial hyperplasia. Antiprogestins such as RU486 are used for blocking ovulation and preventing implantation, and in addition they are in clinical testing for the induction of labor and to control various neoplastic transformations.

Selective progesterone receptor modulators (SPRM) (mesoprogestins) are PR ligands with agonistic and antagonistic activities. Some SPRM show weak antiglucocorticoid or mixed androgenic/antiandrogenic activities. SPRM are currently tested and may be useful, e.g., for the treatment of endometriosis.

Drugs

Pure and partial agonists and antagonists (see individual SHR) are therapeutically used for contraception, hormonal ablation in breast and prostate cancer, and HRT in osteoporosis.

Contraceptives Gluco-Mineralocorticoid Receptors Selective Sex Steroid Receptor Modulators

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Silent Information Regulator 2 (Sir2) Proteins

► Sirtuins

Sirtuins

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Synonyms

Histone deacetylases; Silent information regulator 2 (Sir2) proteins

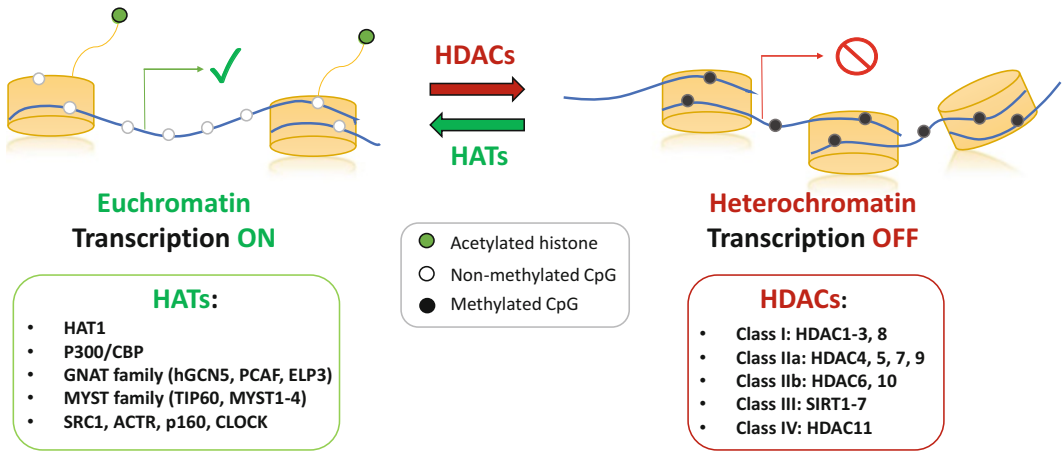
Definition

Histone lysine residues are modulated by post-translational modifications (PTMs) that specifically regulate the three-dimensional structure of chromatin as well as its accessibility to chromatin remodeling complexes and transcriptional factors, thus controlling the entire mechanism of gene expression. Acetylation pattern results from the balanced activities of two classes of epigenetic enzymes known as histone acetyltransferases (HATs) and histone deacetylases (HDACs) that, respectively, add (from acetyl-CoA) and remove the acetyl group from the ε-amino group of specific histone and non-histone lysine residues (Carafa et al. 2016; Schiedel et al. 2018b; Zwergel et al. 2019). HDAC enzymes have been grouped in four different classes, known as I, II, III, and IV, and based on sequence homology to their yeast orthologues Rpd3, HdaI, and Sir2. In particular, to the class III HDACs belong the silent information regulator 2 (Sir2) proteins also known as Sirtuins, which differ from classes I, II, and IV HDACs mainly because of their mechanism of action, which is nicotinamide adenine dinucleotide (NAD^+)- instead of Zn^{2+}-dependent (Carafa et al. 2016; Schiedel et al. 2018b; Zwergel et al. 2019).

► Histone acetylation always correlates with the activation of gene expression and transcription, thanks to the electrostatic change that this process entails at the chromatin level: the introduction of an acetyl group neutralizes the physiologically positive charge of the ε-amino group of histone lysine residues, thus counteracting the electrostatic interaction with the negatively charged DNA phosphate groups (Fig. 1) (Shahbazian and Grunstein 2007).

Sirtuins are involved in numerous biological processes such as cell survival and metabolism, aging, and DNA repair; thus they modulate crucial metabolic pathways.

The seven members of the Sirtuin family are more and more seen as potential targets for the treatment of cancer, metabolic, neurodegenerative, and cardiovascular diseases.



Sirtuins, Fig. 1 Illustration of the euchromatin/heterochromatin switch as a result of the HATs/HDACs balance

Basic Characteristics of Sirtuins

The Sirtuin family consists of seven distinct isoforms showing specific cellular localization, substrate, and catalytic activities which correlate with different physiopathological roles (Table 1) (Kupis et al. 2016).

In fact, despite that Sirtuins are mainly known for their deacetylating activity toward histone and non-histone proteins, such enzymes were firstly identified as ADP-ribosyltransferases, and, subsequently, also the capability to remove acyl moiety different from the simple acetyl group, including lipoyl, malonyl, glutaryl, 3-hydroxy-3-

Sirtuins, Table 1 SIRT1–7 characterization in terms of localization, substrates, catalytic activity, functions, and relative pathological roles

Sirtuin	Localization	Substrates	Catalytic activity	Functions	Pathology
SIRT1	Nucleus, cytosol	PGC1α, eNOS, FOXO, NF-kB, MyoD, PPAR α, H1K26ac, H3K9ac, H4K16ac	NAD^+-dependent protein deacetylation	Cell survival, insulin, signaling, inflammation, metabolism regulation, lifespan regulation, oxidative stress response	Colon, prostate, ovarian, lung, and breast cancer, glioma, melanoma, lung adenocarcinoma, acute myeloid leukemia (AML); neurodegenerative diseases
SIRT2	Nucleus, cytosol	H3K56ac, H4K16ac, α-tubulin, Foxo3a, p53, G6PD, MYC	NAD^+-dependent protein deacetylation, demyristoylation	Cell cycle regulation, nervous system development	Glioma; neurodegenerative diseases
SIRT3	Nucleus, mitochondria	AceC2, ShdhA, SOD2, PDMC1a, IDH2, GOT2, FoxO3a	NAD^+-dependent protein deacetylation	Regulation of mitochondrial energetic metabolism	B-cell chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), breast and gastric cancer; neurodegenerative diseases
SIRT4	Mitochondria	GDH, MCD, PDH	NAD^+-dependent protein deacylation (acyl, lipoyl, and 3-hydroxy-3-methyglutaryl) and ADP-ribosylation	Regulation of mitochondrial energetic metabolism, fatty acid oxidation, and insulin secretion	Breast, colorectal cancer and esophageal squamous cell carcinoma (ESCC)
SIRT5	Mitochondria	Histone H4, CPS1, cyt c	NAD^+-dependent deacylation (malonyl, succinyl, and glutaryl)	Urea cycle and apoptosis regulation	Breast, pancreatic, non-small cell lung carcinoma
SIRT6	Nucleus	H3K9ac, H3K56ac, PARP1	NAD^+-dependent protein deacylation (acyl and long-chain fatty acyl) and ADP-ribosylation	Genome stability, DNA repair, nutrient-dependent metabolism regulation	Breast and colon cancer
SIRT7	Nucleus	RNA pol I, p53, H3K18ac	NAD^+-dependent protein deacetylation	Regulation of rRNA transcription and cell cycle	Liver, spleen, testis, thyroid, and breast cancer

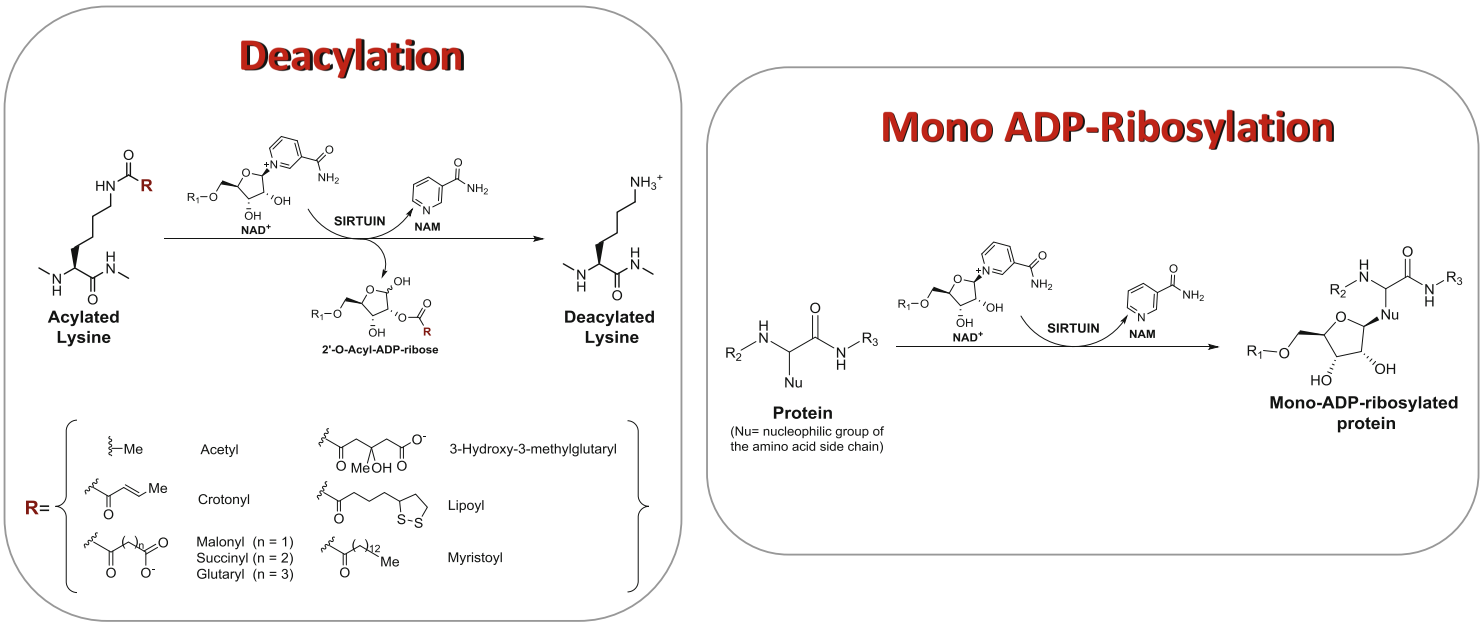
methylglutaryl, succinyl, crotonyl, fatty acyl, etc., has been highlighted (specifically for SIRT2,4–6). Given their mechanism of action (Fig. 2), Sirtuins can be described as metabolism sensors sensitive to cellular NAD^+/nicotinamide (NAM) cellular levels (Kupis et al. 2016). In particular, NAM acts as an endogenous inhibitor of their activity.

All Sirtuins show a conserved catalytic NAD^+-binding site, which consists of about 275 amino acids framed in between an *N*- and *C*-terminal sequences of different length susceptible to post-translational modifications, responsible for variability in their functions and localization (Fig. 3).

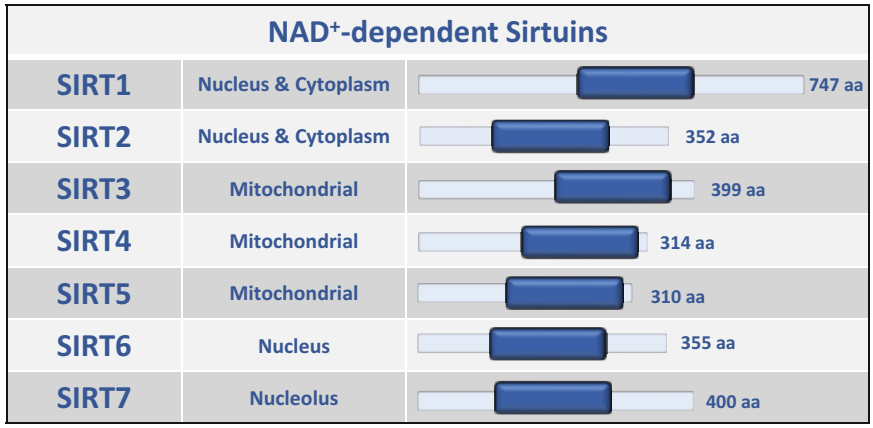
In addition to the essential α/β Rossmann-fold domain, which is typical of NAD^+-binding protein, Sirtuins are also characterized by a Zn^{2+}-binding domain that is composed of three stranded antiparallel β-sheets and a variable α-helical region. The loop responsible for the NAD^+ binding linked the Rossmann-fold with the Zn^{2+}-binding domain and is characterized by four loops, thus generating an extended cleft that forms the enzyme's active site. As previously mentioned, despite that the zinc ion does not take part into the catalysis since it is located too far from the active site, it results crucial for the structural stability of Sirtuins coordinating four different conserved cysteine residues and keeping the three β-strands together (Moniot et al. 2012). Despite that structural rearrangements at the base of the dynamic structure of this class of enzymes have been discovered, individual Sirtuins are characterized by specific preference(s) in sequence related to variables in their electrostatics properties and peptide-binding groove shape. Also, the capability of Sirtuins to recognize a huge number of substrate moieties proves their high adaptability.

Physiopathological Characteristics of Sirtuins

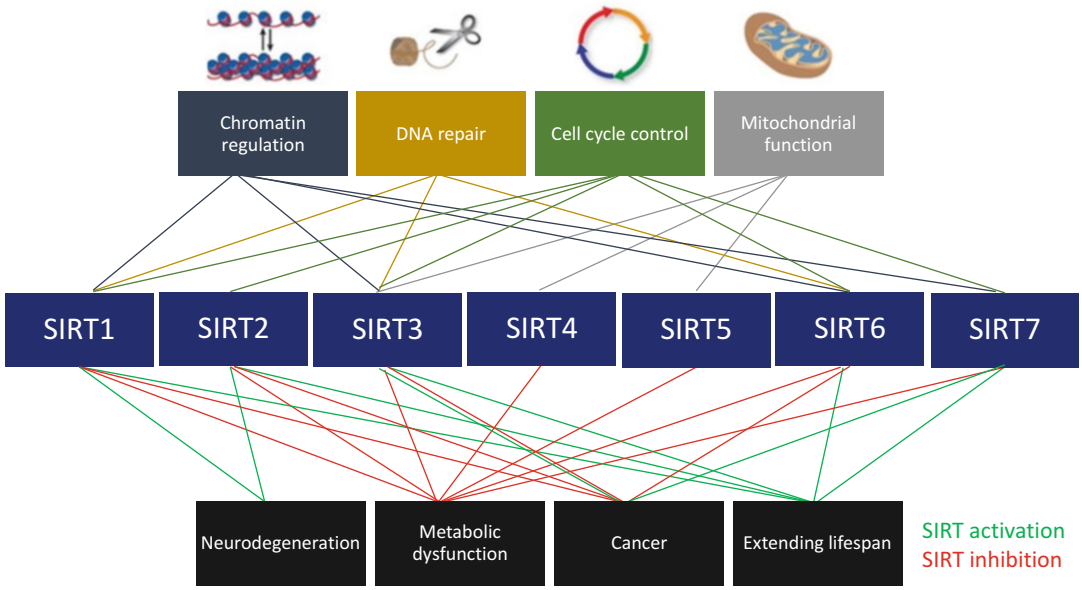
As previously mentioned, Sirtuins activities do not involve only histone substrates but also non-histone targets directly or indirectly related to chromatin regulation, apoptosis, stress response, or differentiation including p53, *c*-Fos, nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), α-tubulin, BubR1, E2F1, tumor necrosis factor-α (TNFα), *c*-Myc, Forkhead box class O (FOXO) factors, hypoxia-inducible factors (HIFs), as well as other epigenetic proteins such as HATs, including p300 and males absent on the first (MOF), the histone methyltransferase (HMT) Enhancer of zeste homolog 2 EZH2, and DNA methyltransferase 1 (DNMT1) (Martinez-Redondo and Vaquero 2013). Sirtuins are implicated in the regulation of a huge variety of crucial processes including transcription, metabolism, aging, cell homeostasis, senescence, apoptosis, and ► inflammation. Several pieces of evidence proved their connection with the onset and progression of metabolic (obesity and diabetes), neurodegenerative (Alzheimer's (AD), Huntington's (HD), and Parkinson's diseases (PD)), and inflammatory diseases (psoriasis) as well as with both hematological (B-cell chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML)) and solid tumors (breast, colon, lung, prostate cancer, etc.); (see Fig. 4) (Carafa et al. 2016; Schiedel et al. 2018b; Zwergel et al. 2019). SIRT1 is the first SIRT family member to be identified and is the isoform for which the most exhaustive information has been collected to date. Through its deacetylase activity, SIRT1 activates the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC1α) and FOXO1, thus promoting gluconeogenesis, whereas it inhibits glycolysis via the repression of the glycolytic enzyme phosphoglycerate mutase 1 (PGAM-1). In addition, liver kinase B1 (LKB1) SIRT1-mediated deacetylation in the skeletal muscle activates ► AMP-activated protein kinase (AMPK), thus establishing a reciprocal positive regulating loop in which AMPK in turn activates SIRT1 enhancing the level of NAD^+, thanks to the upregulation of NAD^+ synthetic enzyme nicotinamide phosphoribosyltransferase (NAMPT) encoding genes. While SIRT1 seems to exert a neuroprotective role modulating the expression and survival of brain-derived neurotrophic factor (BDNF), it also correlates with tumorigenesis leading to the inhibition of p35 and p73 via their deacetylation and to the activation of the oncoprotein BCL6, thus favoring an antidifferentiating and anti-apoptotic effect through the deacetylation of different



Sirtuins, Fig. 2 Schematization of Sirtuins mechanisms of action: on the left is represented the diacylation, and on the right the ADP-ribosylation process



Sirtuins, Fig. 3 Schematic representation of human Sirtuins in which the shared NAD^+-catalytic domain is highlighted in blue



Sirtuins, Fig. 4 Schematic representation of the involvement of SIRT isoforms in various diseases and their possible modulation

transcription factors (FOXO3a, Ku70, E2F1, etc.). Despite that an overexpression (OE) of SIRT1 has been registered in different kind of chemotherapy-resistant tumors such as prostate, breast, liver, colorectal, ovarian, melanoma, and epithelial cancer, also a downregulation of such Sirtuin correlates with malignancies (glioblastoma, bladder and colon cancer). SIRT1 functions within the oncological scenario seem to be context specific since SIRT1 modulates the expression of various DNA repair and tumor suppressor genes.

SIRT2 is principally localized in the cytoplasm and is responsible for the deacetylation of the Lys40 of α-tubulin, but several data showed that, during cell cycle progression from phase G_2 to M, it moves toward the nucleus providing the deacetylation of H4K16 which correlates with metaphasic heterochromatin. In addition, also

p300, FOXO1, FOXO3, and glucokinase regulatory protein (GKRP) have been identified as other targets of its deacetylase activity. Glucokinase (GCK) is crucial for retaining the glucose homeostasis modulated by the binding of GKRP, which, in its acetylated state, correlates with diabetes mellitus. In addition, other evidence emphasized the role covered by SIRT2 in other metabolic processes, such as adipogenesis. Unlike SIRT1, SIRT2 possesses a neurodegenerative role in neurological pathologies, and it modulates cell progression and mitotic and apoptotic events displaying both tumor promoter and suppressor capability.

SIRT3, together with SIRT4 and SIRT5, is one of the three mitochondrial Sirtuins. Specifically, SIRT3 takes part in the modulation of mitochondrial activities by providing the deacetylation and consequent activation of various electron transport chain complexes (I and II) components as well as acetyl-CoA synthetase (ACS). SIRT3 is known to be involved in the counteraction of the oxidative stress protecting cells from reactive oxygen species (ROS) in microglia and activating, under calorie restriction condition, the essential mitochondrial ► antioxidants actor superoxide dismutase 2 (SOD2) in cochlea neurons, thus playing an overall neuroprotective role. SIRT3 controls differentiation, proliferation, and survival of cells acting as a tumor suppressor through the deacetylation and activation of pyruvate dehydrogenase (PDH) that correlates with the repression of glycolysis metabolism. However, also the role covered by SIRT3 in tumors still needs to be clarified.

Until recently, SIRT4 was the less-investigated Sirtuin, and it was firstly described for its ADP-ribosyltransferase activity toward glutamate dehydrogenase (GDH). Effects of SIRT4 are a reduced amino-acid-stimulated insulin secretion in pancreatic B-cells and an increased genome stability as a consequence of the SIRT4-mediated inhibition of glutamine anaplerosis, a crucial step for an effective cell cycle arrest after DNA damage. Thus, SIRT4 seems to act as a tumor suppressor, but, in this framework, further investigations are necessary since the delayed cell cycle in DNA-damaging condition mediated by SIRT4 can also reduce the sensitivity of tumors cells to cytotoxic treatment. As proof of this, overexpression of SIRT4 correlates with increased mortality rate during cisplatin treatment (Jeong et al. 2016). SIRT4 inhibits the PDH via its lipoamidation and shows a weak deacetylase activity toward the malonyl Co-A decarboxylase (MCD), which is the main way, in addition to the indirect interaction with ANT2 and AMPK, whereby SIRT4 represses the β-fatty acid oxidation. SIRT4 also mediates the sensitivity to cardiac performance to hypertrophic stress modulating the activity of manganese superoxide dismutase (MnSOD). Recently, Pannek et al. reported the 3-hydroxy-3-methylglutaril (HMG) residue as new acyl toward which SIRT4 shows an efficient enzymatic activity solving also the crystal structure of SIRT4 from *Xenopus tropicalis* (xSIRT4, 81% similarity with human SIRT4 (hSIRT4)) (Pannek et al. 2017).

SIRT5 is mainly expressed in the heart, liver, kidney, and brain and exhibits as main targets the restricting enzyme of the urea cycle carbamoyl synthetase 1 (CPS1) and poly-(ADP-ribose) polymerase 1 (PARP1), which is responsible for the stimulation of DNA repair mechanisms upon oxidative-induced damage, toward which SIRT5 acts as a deacetylase and ADP-ribosyltransferase, respectively. Despite that SIRT5 OE was detected in non-small cell lung carcinoma (NSCLC), overlapping with poor prognosis, further evidence is required to explain in a comprehensive way its role in cancer.

SIRT6 catalyzes the removal of fatty acyl moiety from Lys19 and Lys20 of TNF-α regulating its release. SIRT6 controls metabolism, cellular homeostasis, telomere maintenance, and DNA repair and for these reasons has been defined as "epigenetic guardian of cellular differentiation."

The last component of this family, SIRT7 thanks to its capability to mediate the deacetylation of H3K18ac leading to the malignant transformation marker H3K18, is being considered a promising and challenging target in the context of the epigenetic fight against cancer. OE levels of SIRT7 were typically registered in cancers with poor prognosis, but also its depletion is associated with aggressive tumors. Also, non-

histone interactions have been identified for this protein such as those with HIF-1α and HIF-2α, thus reducing their expression.

Sirtuin Modulators

Sirtuin modulators are today applied alone or in combination with either approved drugs or other epigenetic agents. They have been investigated for the treatments of neurodegeneration or metabolic diseases as well as various types of cancer (Villalba and Alcain 2012; Schiedel et al. 2018b). Table 2 is summarizing all the SIRT modulators discussed herein.

Sirtuin Inhibitors (SIRTi)

Small Molecules

The first SIRTi splitomicin (**1**) appeared in literature in 2001 and was identified via a yeast-based phenotypic screening. This compound was inactive against human Sirtuins but was the initial point for the discovery of numerous human SIRT1/2i (Carafa et al. 2016; Zwergel et al. 2019).

One important splitomicin derivative is the SIRT1i HR-73 (**2**) able to block the HIV transcription via TAT protein deacetylation (Carafa et al. 2016; Zwergel et al. 2019).

EX-527 or selisistat **3**, discovered in 2005, was the first potent and selective cell-permeable SIRT1i. Even if this compound increased p53 acetylation levels, only poor effects were observed in cancer. Compound **3** is now being investigated with promising results in Huntington's disease also in a clinical context. Additionally, **3** blocked via a SIRT1 inhibition-mediated mechanism the viral amplification of human papillomavirus (Carafa et al. 2016; Zwergel et al. 2019).

Cambinol (**4**) is a moderate SIRT1/2i, and its treatment led to hyperacetylated p53, α-tubulin, FOXO3a, and Ku70 in lymphoma and HeLa cancer cells decreasing the tumor burden also in xenograft mice (Carafa et al. 2016; Zwergel et al. 2019). Auspicious anticancer features were also described for hepatocellular cancers and neuroblastoma (Carafa et al. 2016; Zwergel et al. 2019). Following studies resulted in various optimized derivatives with improved potency and/or selectivity for either SIRT1 or SIRT2. One example of such a strategy is MC2141 (**5**) (Carafa et al. 2016; Zwergel et al. 2019).

AGK2 (**6**) possesses a selectivity against SIRT2 over SIRT1/3, being a low micromolar SIRT2i able to maintain the acetylation of α-tubulin in HeLa cells. Compound **6** rescues dopaminergic neurons from α-synuclein toxicity in Parkinson's disease and leads to caspase-3-dependent apoptosis in glioma cells (Carafa et al. 2016; Zwergel et al. 2019).

Tenovin1 (**7a**) and its water-soluble derivative (**7b**) are micromolar SIRT1-3i able to activate p53 in melanoma, leukemia, and gastric cancer in vitro and in vivo (Carafa et al. 2016; Zwergel et al. 2019).

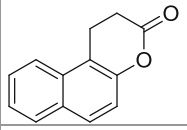
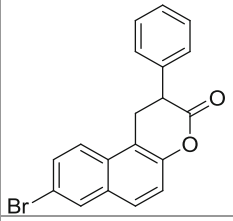
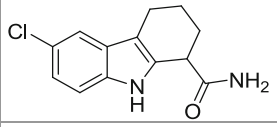
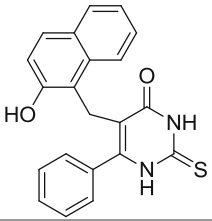
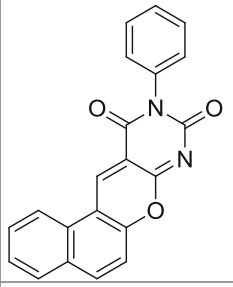
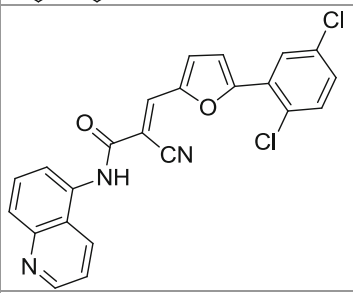
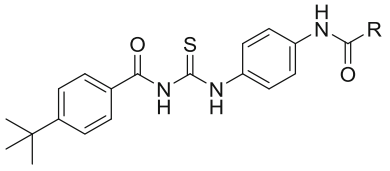
Also, sirtinol (**8**), a SIRT1/2i, identified in 2001 via a high-throughput screening, possesses numerous anticancer effects in human MCF7 and H1299 cells as well as in adult T-cell leukemia/lymphoma (ATL). Furthermore **8** increases the chemosensitivity to cisplatin and camptothecin in PC3 and HeLa cells (Carafa et al. 2016; Zwergel et al. 2019).

Salermide (**9**) is an optimized form of compound **8**. Besides the numerous anticancer effects through SIRT1 inhibition which result in the H4K16ac deacetylation and ultimately in the repression of pro-apoptotic genes such as *CASP8*, *TNF*, *TNFRSF10B*, and *PUMA*, salermide treatment also protects muscle cells in a dystrophy model (Pasco et al. 2010). Interestingly, **9** has beneficial effects upon *Schistosoma mansoni* (Sm) infections via *Sm*SIRT inhibition leading to apoptosis, death of schistosomula, and reduced egg laying (Carafa et al. 2016; Zwergel et al. 2019).

Subsequently, selective SmSIRT2i were identified from a commercial library and afterward optimized. Compound **10** possesses a good efficacy against larval and adult worms without affecting human Sirtuins and human cells (Monaldi et al. 2019).

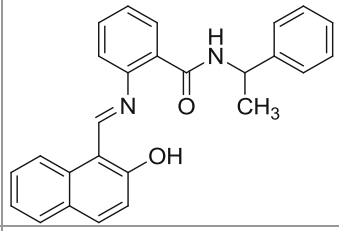
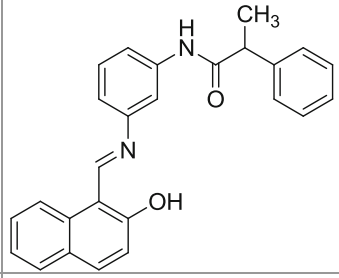
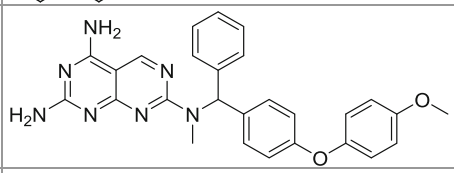
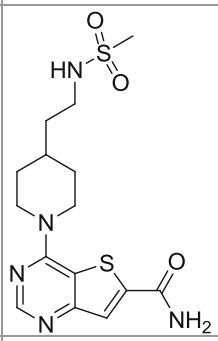
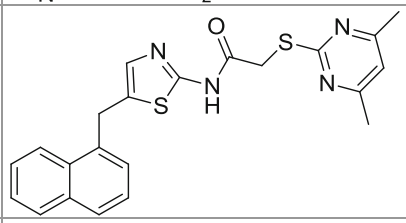
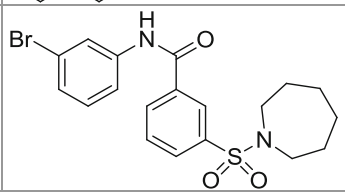
Compound **11** and its analogues are one of the most potent SIRTi discovered so far. Even though the crystal structure has been solved and a

Sirtuins, Table 2 Most relevant sirtuin modulators

Most relevant sirtuin inhibitors		
Compound	Structure	Enzyme activity
1, Splitomicin		ySir2 $IC_{50} = 60\ \mu M$ SIRT1: No inhibition at 500 μM
2, HR-73		SIRT1 $IC_{50} = <5\ \mu M$
3, EX-527, selisistat		SIRT1 $IC_{50} = 0.098\ \mu M$ SIRT2 $IC_{50} = 19.6\ \mu M$ SIRT3 $IC_{50} = 48.7\ \mu M$
4, Cambinol		SIRT1 $IC_{50} = 56\ \mu M$ SIRT2 $IC_{50} = 59\ \mu M$ SIRT3: No inhibition at 300 μM SIRT5: 42% inhibition at 300 μM
5, MC2141		SIRT1 $IC_{50} = 9.8\ \mu M$ SIRT2 $IC_{50} = 12.3\ \mu M$
6, AGK2		SIRT1 $IC_{50} > 50\ \mu M$ SIRT2 $IC_{50} = 3.5\ \mu M$ SIRT3 $IC_{50} > 50\ \mu M$
7a, tenovin-1 7b, tenovin-6	 **7a** R = CH_3 **7b** R = $(CH_2)_4N(CH_3)_2$ HCl	**7a**: IC_{50}s not determined due to lack of water solubility **7b**: SIRT1 $IC_{50} = 21\ \mu M$ SIRT2 $IC_{50} = 10\ \mu M$ SIRT3 $IC_{50} = 67\ \mu M$

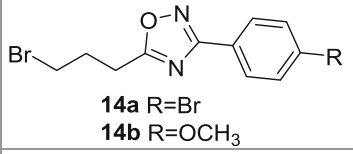
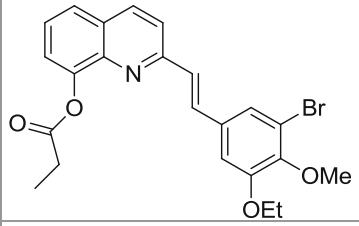
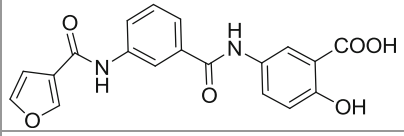
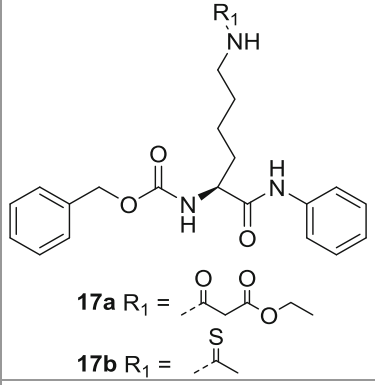
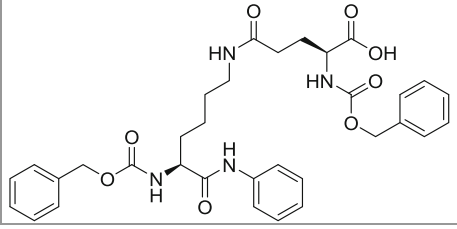
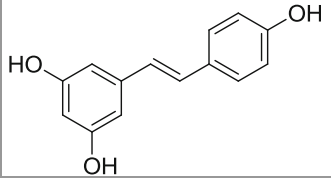
(continued)

Sirtuins, Table 2 (continued)

Most relevant sirtuin inhibitors		
Compound	Structure	Enzyme activity
8, Sirtinol		ySir2 $IC_{50} = 70$ μM SIRT1 $IC_{50} = 131$ μM SIRT2 $IC_{50} = 49$ μM
9, Salermide		SIRT1 $IC_{50} = 43$ μM SIRT2 $IC_{50} = 25$ μM
10		*Sm*SIRT2 $IC_{50} = 2.3$ μM hSIRT2 $IC_{50} = 22.1\%$ at 25 μM
11		SIRT1 $IC_{50} = 4$ nM SIRT2 $IC_{50} = 1$ nM SIRT3 $IC_{50} = 7$ nM
12, SirReal2		SIRT2 $IC_{50} = 0.14$–0.44 μM SIRT1 22% inhibition at 100 μM SIRT3 no inhibition at 100 μM SIRT4 no inhibition at 200 μM SIRT5 no inhibition at 200 μM SIRT6 19% inhibition at 200 μM
13, AK-7		SIRT2 $IC_{50} = 15.5$ μM SIRT1/3 no inhibition at 50 μM

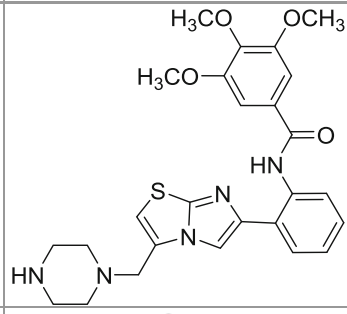
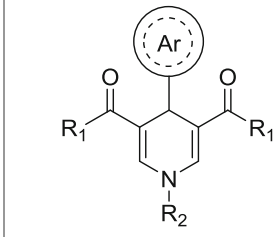
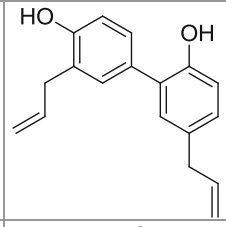
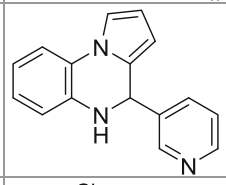
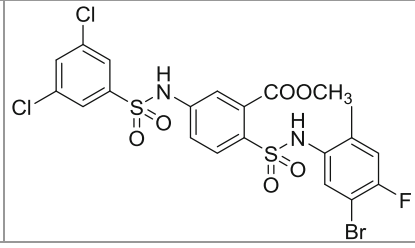
(continued)

Sirtuins, Table 2 (continued)

Most relevant sirtuin inhibitors		
Compound	Structure	Enzyme activity
14a, 14b	 **14a** R=Br **14b** R=OCH_3	**14a**, SIRT2 IC_{50} = 1.5 μM **14b**, SIRT2 IC_{50} = 10.4 μM Inactive at 100 μM against SIRT1, SIRT3, and SIRT5
15, SDX-437		SIRT3 IC_{50} = 700 nM SIRT1 no inhibition at 100 μM
16		SIRT6 IC_{50} = 89 μM SIRT1 IC_{50} = 1578 μM SIRT2 IC_{50} = 751 μM
17a, 17b		**17a** SIRT1 IC_{50} = 3.9 μM **17b** SIRT1 IC_{50} = 2.7 μM SIRT2 IC_{50} = 23 μM SIRT3 IC_{50} > 100 μM
18, MC3482		SIRT5 42% at 50 μM No inhibition for other isoforms at 50 μM
Most relevant Sirtuin activators		
19, Resveratrol		SIRT1 $EC_{1.5}$ (effective concentration able to increase the enzyme activity of 150%) = 46.2 μM

(continued)

Sirtuins, Table 2 (continued)

Most relevant sirtuin inhibitors		
Compound	Structure	Enzyme activity
20, SRT1460		SIRT1 $EC_{1.5} = 2.9$ μM SIRT1 max act. 447%
21, DHPs	 Ar= aryl, heteroaryl R_1= OEt, OH, NH_2 R_2= benzyl, benzoyl, acyl, etc.	For Ar = Ph, R_1 = OEt, R_2 = benzyl: SIRT1 $EC_{1.5} \approx 1$ μM SIRT2 $EC_{1.5} = 25$ μM SIRT3 $EC_{1.5} \approx 50$ μM
22, Honokiol		Increases SIRT3 levels by nearly twofold at 5 μM and 10 μM in cardiomyocytes after 24-h treatment In vitro directly binds to SIRT3 and increases the affinity of SIRT3 for NAD^+
23, MC3154		Increases SIRT6 levels in a dose-dependent manner (max. Twofold) $EC_{50} = 38$ μM
24, MDL-800		Increase of SIRT6 deacetylation activity by >22-fold at 100 μM $EC_{50} = 10.3$ μM

nanomolar biochemical activity against SIRT1–3 has been observed, no biological activity has yet been described (Carafa et al. 2016; Zwergel et al. 2019).

SirReal2 (**12**) is a highly selective sub-micromolar SIRT2i (>1000 fold over all SIRT isoforms) (Carafa et al. 2016; Zwergel et al. 2019).

Crystallography studies revealed a previously unexploited SIRT2-binding mode inducing a structural rearrangement in the active site of SIRT2. Hyperacetylation of α-tubulin and destabilization of the SIRT2 substrate BubR1 upon SirReal2 treatment confirmed the involvement of SIRT2 inhibition in HeLa cells. Recently the SirReal hybrid compounds with thalidomide were created to apply the proteolysis-targeting chimeras (PROTACs) approach, being the first example of chemical induction of SIRT2 degradation (Schiedel et al. 2018a).

Another micromolar, selective, and brain-penetrating SIRT2i, AK-7 (**13**), possesses SIRT2-dependent neuroprotective properties in neurodegenerative diseases such as HD and PD; furthermore it is capable to downregulate the neuronal cholesterol biosynthesis, confirming the crucial role of SIRT2 as a target in neurodegenerative disorders (Carafa et al. 2016; Zwergel et al. 2019).

SIRT2i can be applied for cancer treatment as well. The compounds **14a** and **14b** out of a 1,2,3-oxadiazole series are selective single-digit micromolar SIRT2i (inactive at 100 μM against Sirt1, Sirt3, and Sirt5) able to induce pro-apoptotic and antiproliferative effects in diverse leukemia cell lines (Carafa et al. 2016; Zwergel et al. 2019).

An example of a selective submicromolar SIRT3i has been described SDX-437 (**15**); however biological data are not yet available (Carafa et al. 2016; Zwergel et al. 2019).

The first SIRT6i **16** was found through an in silico high-throughput screening approach, a greater than eightfold selectivity over SIRT1/2 (Carafa et al. 2016; Zwergel et al. 2019). This compound might be a promising first step toward optimized SIRT6i and an eventual therapeutic application, since it hyperacetylates the SIRT6 substrate H3K9, and increases glucose uptake via GLUT-1 upregulation

Peptides and Pseudopeptides

Besides small molecules, also peptide-based SIRTi are described in literature. In the first one, the N^{ε}-acetyl-lysine residue was substituted with a thioacetylated moiety, and the *C*-terminal was prolonged with a p53 protein tail (Schiedel et al. 2018b), resulting in a SIRT1/2i with also a moderate effect on SIRT3. Thereafter other quite potent thioacetylated peptide-based inhibitors as well as other lysine N^{ε}-modifications (such as propionyl, α-hydroxyacetyl, homocitrulline, or homoarginine residues) were synthesized and tested resulting in the inhibition of SIRT1/2/3 using different SIRT substrates such as human α-tubulin or acetyl-coenzyme A synthetase 2 (AceCS2) (Schiedel et al. 2018b).

Most of the peptidic or pseudopeptidic SIRTi (**17a, 17b**) reported to date are SIRT1/2/3i; nevertheless some of them were slight active also toward SIRT6 (Carafa et al. 2016; Zwergel et al. 2019) or SIRT5 (Rajabi et al. 2017; Kalbas et al. 2018).

A specific peptide-based SIRT5i MC3482 (**18**) has been shown to increase ammonia production in non-liver cells leading to autophagy via modulation of the glutamine metabolism (Zwergel et al. 2019).

SIRT Activators

Besides the above presented SIRTi, also SIRT activators (SIRTa) have been described in literature (Schiedel et al. 2018b). The first and most prominent example is the natural polyphenol resveratrol (**19**), a SIRT1a. This compound can extend the lifespan of rather simple organisms such as yeasts, worms, fishes, and insects. Later on these promising outcomes, **19** was also published to improve mitochondrial functions in mammals such as normal and obese rodents (Carafa et al. 2016; Zwergel et al. 2019).

The selective SIRT1a SRT1460 (**20**) was identified via a high-throughput screening. Interestingly, it shows similar effects like **19** in vivo however associated with a higher potency (Carafa et al. 2016; Zwergel et al. 2019). Compound **20** and its analogues exhibited encouraging results in preclinical studies for age-related diseases; thus some of them are now evaluated in clinical trials (Carafa et al. 2016; Zwergel et al. 2019).

In the past, SIRT1 activation was much debated due to the fact that the initial studies had positive outcome only with artificial substrates such as carboxytetramethylrhodamine (TAMRA)-

labeled ones, whereas SIRT1 activation was only recently confirmed by the delicate structural and positional requirements of SIRT1 in the presence of its natural substrates such as FOXO3a and PGC-1α (Carafa et al. 2016; Zwergel et al. 2019) and by the crystal structure of an engineered human SIRT1-sirtuin-activating compound (STAC) complex, which revealed the STAC-binding site within the *N*-terminal domain of hSIRT1 (Dai et al. 2015).

1,4-Dihydropyridines (DHPs) of the general structure **21** are SIRT1a able to decrease the acetylation state of α-tubulin in U937 cells, to increase the NO release in HaCat cells, and to improve wound healing and skin repair in a mouse model via the activation of the SIRT1/AMPK pathway. Additionally, a water-soluble DHP analogue increased H4K16ac deacetylation resulting in antiproliferative effects in diverse cancer cell lines at a micromolar level (Carafa et al. 2016; Zwergel et al. 2019).

SIRT3 can be directly activated and overexpressed by the natural compound honokiol (**22**) resulting in reduced acetylation levels of the mitochondrial SIRT3 substrates MnSod or oligomycin-sensitivity conferring protein (OSCP). The compound **22** showed beneficial properties in a mouse model of hypertrophy via SIRT3 activation (Carafa et al. 2016; Zwergel et al. 2019).

Compound **23** is the first small molecule identified as SIRT6a. The biochemical and crystallographic data revealed a direct, specific, and substrate-independent binding mode of **23** within the catalytic site of SIRT6 and its subsequent potent activation which results in the deacetylation of peptide substrates and entire nucleosomes (Zwergel et al. 2019). The possibility of SIRT6 activation by small molecules has been recently further confirmed by a more potent compound **24** (Huang et al. 2018).

Cross-References

- ▶ AMP-Activated Protein Kinase
- ▶ Antioxidants
- ▶ Histone Acetylation
- ▶ Inflammation

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Skeletal Metabolism

► Bone Metabolism

Skeletal Turnover

► Bone Metabolism

Small G Proteins

► Small GTPases

Small GTPases

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Synonyms

Low molecular mass GTPases; Small G proteins

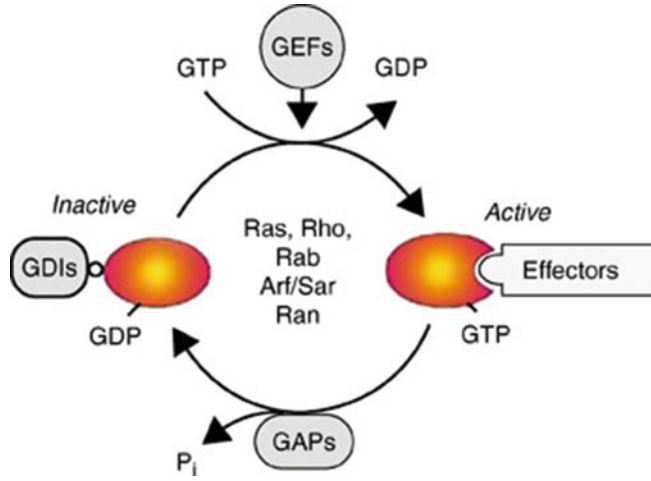
Definition

Small GTPases are monomeric 20–40 kD GTP-binding proteins that interconvert between an active (GTP-bound) and an inactive (GDP-bound) state. As molecular switches they are involved in the regulation of complex cellular processes.

Basic Characteristics

Regulation

Activation of small GTPases occurs by GDP/GTP exchange catalyzed by guanine nucleotide exchange factors (GEFs) (Fig. 1). They stimulate the dissociation of GDP in response to an upstream signal which results in binding of GTP. In the GTP-bound form the GTPases are active, and bind to and activate a number of effector molecules. The small G proteins are able to hydrolyze the bound nucleotide to GDP. This inactivation step is accelerated by GTPase activating proteins (GAPs). In the GDP-bound form the GTPases are inactive. Some GTPases bind to guanine nucleotide dissociation inhibitors (GDIs) that stabilize the inactive form and cover the lipid modification of the GTPase forming a cytosolic complex. Novel roles of GDI in GTPase regulation like the delivery of GTPases to specific sites within the cell are discussed. Moreover, local synthesis and degradation of GTPases seem to



Small GTPases, Fig. 1 The GTPase cycle: GTPases are inactive in the GDP-bound form. In a complex with guanine nucleotide dissociation inhibitors (GDIs) the inactive form is stabilized (note: not all small GTPases are regulated by typical GDIs, e.g., not Ras, Ran, and Arf subfamily proteins). Guanine nucleotide exchange factors (GEFs) cause the release of GDP and binding of GTP, and thereby the activation of the proteins. The active state of the GTPases is turned off by GTP hydrolysis catalyzed by GTPase activating proteins (GAPs)

play additional roles in the spatial regulation of small GTPases. In mammalian cells, each family of the regulating proteins GAPs, GEFs, and GDIs comprise numerous members that are more or less specific for individual GTPases, cell types, GTPase functions, and signaling pathways.

General Structural Properties

All small GTPases are folded in a similar way. They possess four consensus amino acid sequences in common, which are involved in nucleotide binding and hydrolysis: GXXXXGK, DXXG, NKXD, and EXSAX. Two highly flexible regions (Switch I and Switch II regions) determine the nucleotide-dependent activation state of the GTPases and the protein–protein interactions with effectors and regulatory proteins.

Posttranslational Modification

All small GTPases (except Ran) are posttranslationally modified. Most important is the isoprenylation of the C-terminus. The type of modification is determined by the COOH-terminal amino acid sequence. GTPases with a C-terminal CAAX-box (A = aliphatic amino acid, X = any amino acid) are farnesylated at the cysteine residue followed by the proteolytic degradation of the last three amino acids and subsequent methylation of the carboxy-terminus. In the case of CAAL or CAC, the cysteines are modified by geranylgeranylation. In some cases an additional cysteine is palmitoylated or N-terminal myristoylation occurs. All these post-translational modifications allow the interaction of GTPases with the phospholipid bilayer. Lipid modification of the GTPases is required for membrane localization and GDI binding.

Families

The superfamily of small GTPases consists of more than 100 members from yeast to human with more than 80 members expressed in mammalian cells. Based on structural and functional similarities the GTPases are subdivided into five major classes.

Ras GTPases

The mammalian family of Ras GTPases consists of more than 15 members, which share high homology to each other and include Ha-Ras, Ki-Ras, N-Ras, R-Ras, Rap, Ral, Rheb, Rin, and Rit proteins (Hancock 2003). Ras proteins have achieved attention with the discovery that they contain point mutations in 15% of all human

tumors (more than 90% in pancreatic tumors), leading to the exchange of conserved amino acids, for example, at positions 12 and 61. Amino acid exchanges at these positions block the GTP hydrolyzing capacity of the GTPases, resulting in constitutive activation. Ras GTPases are involved in proliferation and/or differentiation. They couple receptor tyrosine kinases with a cascade of cytosolic kinases termed Raf/ERK kinase pathway (also known as MAP kinase cascade). Activation of this pathway leads to phosphorylation and activation of transcription factors like Elk-1, and stimulate gene expression. Activated Ras has been shown to transform culture cells and to produce tumors in nude mice. Besides the Raf kinase, also RalGDS, which is an activator of the Ral subfamily proteins, and the PI3 kinase involved in inositol signaling are important effectors of Ras signaling. Ral GTPases (<50% identical with Ras) control cell proliferation, Ras-mediated cell transformation, vesicle traffic, phospholipase D, and cytoskeleton organization. Rap GTPases have been identified in a screen for cDNAs that are able to revert the transforming phenotype of Ki-Ras (Kirsten Ras) and, therefore, were also termed K-rev proteins.

Rho GTPases

Members (>20) of the Rho family of GTPases, including RhoA, B and C, Cdc42 and Rac1, 2 and 3, share more than 50% sequence identity. These GTPases are important regulators of the actin cytoskeleton (Etienne-Manneville and Hall 2002). RhoA regulates the formation of actin stress fibers, whereas Cdc42 is known to induce filopodia. Rac is involved in the formation of lamellipodia and membrane ruffles. Rho GTPases are involved in migration, phagocytosis, endo- and exocytosis, and cell–cell and cell–matrix contact. Rac regulates NADPH oxidase. Furthermore, Rho GTPases are involved in transcriptional activation, cell transformation, and apoptosis. Considering their diverse functions, Rho GTPases are regulated by a large number of GEFs and GAPs (>60 members of each family have been identified), suggesting spatiotemporal, function-specific regulation.

A subfamily of Rho proteins, the Rnd proteins are always GTP-bound and seem to be regulated by expression and localization rather than by nucleotide exchange and hydrolysis. Many Rho GTPase effectors have been identified, including protein and lipid kinases, phospholipase D, and numerous adaptor proteins. One of the best characterized effector of RhoA is Rho kinase, which phosphorylates and inactivates myosin phosphatase; thereby RhoA causes activation of actomyosin complexes. Rho proteins are preferred targets of bacterial protein toxins (► Bacterial Toxins).

Rab GTPases

The largest family of small GTPases with more than 40 members identified is the family of Rab GTPases (Zerial and McBride 2001). Rab proteins are important regulators of specific steps of intracellular vesicle trafficking, including budding, targeting, docking, and fusion with acceptor membranes. Each Rab protein has an organelle-specific subcellular localization and seems to be functionally specialized. Rab1A and Rab1B are two of the most extensively studied members of the Rab family. Both proteins are found in membranes of the ER, Golgi apparatus, and intermediate vesicles between these compartments. They appear to function in the anterograde trafficking of proteins from the ER to the Golgi compartment. Rab4 and Rab5 are present on early endosomes and are involved in the endocytic process, whereas Rab6 is localized at the Golgi apparatus regulating processes of the secretory pathway. One of the best studied members of the Rab protein family is Rab3a. This GTPase is a key regulator of Ca^{2+}-induced exocytosis, particularly in nerve terminals. Several effectors of Rab proteins like Rabphilin, Rabaptin, and Rim have been identified and characterized as essential for vesicle trafficking. Recently, the Rab effector Rabkinesin6 has been identified that links Rab proteins to the microtubule cytoskeleton. Rabkinesin6 may be the motor driving vesicles along microtubules from the Golgi apparatus to the periphery.

Arf/Sar1 GTPases

The name Arf (ADP-ribosylation factor) stems from its discovery as a cytosolic factor with the

ability to enhance the ADP-ribosylation of the α-subunit of the G protein G_S by cholera toxin. Arf is known to regulate phospholipid metabolism. Studies with dominant active or dominant negative mutants of Arf proteins in mammalian cells suggest the involvement of these GTPases in the trafficking of coated vesicles, and it is now known that Arf1 regulates the formation of COPI-coated vesicles for retrograde transport between Golgi apparatus and endoplasmic reticulum (Souza-Schorey and Chavrier 2006). Sar1, which is 37% identical to Arf1, is needed for the assembly of COPII proteins for vesicle transport in the opposite direction. Taken together, Arf and Sar proteins play crucial roles in the recruitment of COP components to vesicles thereby regulating vesicle budding. In contrast to the other small GTPases, Arf/Sar1 proteins are not regulated by GDI proteins, whereas different GEF and GAP proteins have been identified. Myristoylation of Arf proteins at the N-terminus is required for its membrane localization.

Ran GTPases

In mammalian cells there is only one *Ran* gene, which was discovered as a Ras-like gene (Ran: Ras-related nuclear protein) (Dasso 2002). In contrast, in yeast more than one related *Ran* genes have been identified. The predominant nuclear localization of the GTPase was the first hint that Ran is involved in nucleocytoplasmic transport processes. Interestingly, the only Ran GEF present in mammalian cells, RCC1 (regulator of chromatin condensation), is localized exclusively in the nucleus, whereas the single Ran GAP (Ran GAP1) is in the cytoplasm. This specialized localization of the regulators is the prerequisite for the asymmetric distribution of the GDP- and GTP-bound form of Ran and for its role as a nucleocytoplasmic transporter. In contrast to other GTPases, the activity of Ran is dependent on the gradient of the GTP-bound GTPase from cytoplasm to nucleoplasm that allows the transport of cargo proteins. Ran is involved in nuclear import as well as in export of proteins through the nuclear pore complex. Both processes require the formation of protein complexes, including Ran, the cargo protein, and Ran-binding proteins like importins or exportins. In addition to its transporter function, Ran has been shown to participate in microtubule organization during the M phase of the cell cycle.

Cascades and Cross-Talk

Small GTPases are not isolated molecular switches. Signaling cascades within one subfamily and cross-talk between members of different subfamilies are known. For example, Cdc42/Rac/Rho is sequentially activated after extracellular stimuli in quiescent Swiss 3T3 cells. Moreover, reciprocal modulation between Rho GTPases has been described. Ras and Rho proteins act in a cooperative manner in Ras-induced transformation. A further example of cross-talk between GTPase families is the cooperative function of Rho and Rab proteins during cell migration, with Rho proteins controlling the actin cytoskeleton, and Rab proteins regulating vesicular traffic for the recruitment of membrane material, and the recycling of proteins like integrins. Arfaptin connects signaling via Arf and Rac in regulating fundamental processes like endocytosis and secretion.

Drugs

Small GTPases, among other activities, regulate cell growth, neurite outgrowth, and signaling of immune cells involved in inflammation. Pharmacological modulation of the activity of small GTPases is thus a useful aim in cancer and anti-inflammatory therapies. Despite many years of scientific work, specific inhibition of Ras is not yet possible; however, the prenyl binding protein PDEdelta sequesters oncogenic Ras within the cytosol to suppress its membrane localization and signaling. Moreover, inhibitors for an activating mutation of the Ras effector Raf are in clinical use. Farnesyltransferase inhibitors block the posttranslational modification of several GTPases, to block correct cellular localization and therefore the transforming activity of Ras or Rho GTPases. Moreover, inhibitors of Rho effectors like Rho kinase inhibitors have been generated.

Cross-References

- ▶ Bacterial Toxins
- ▶ Growth Factors

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Small Molecules

- ▶ Rheumatoid Arthritis
- ▶ Small-Molecule Screens

Small-Molecule Screens

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Synonyms

Chemical biology; Drug screening; High-throughput screening (HTS); Small molecules

Definition

Methods to search for compounds of low molecular weight which modulate biological experiments, typically using libraries of several thousands to millions of small molecules. Robotic automation is typically used for a precise and simultaneous transfer of reagents and biologically active probes such as enzymes or living cells to microtiter test plates. Automated detection systems measure biological responses, such as a decrease of fluorescence through the inhibition of an enzyme by a small molecule. Other studies examine the effects of compounds on proteins or other purified components to measure their interactions or functions. More complex experiments analyze the effects of small molecules on living cells, such as cancer cells from patients, or pluripotent stem cells as they develop into other cell types or organ-like structures. Screens may also be based on animal models such as C. *elegans* or zebrafish embryos. Identifying small molecules that modulate biological functions has become crucial to studying molecular mechanisms in health and disease, validating novel drug targets, and developing novel diagnostics and drugs.

Description

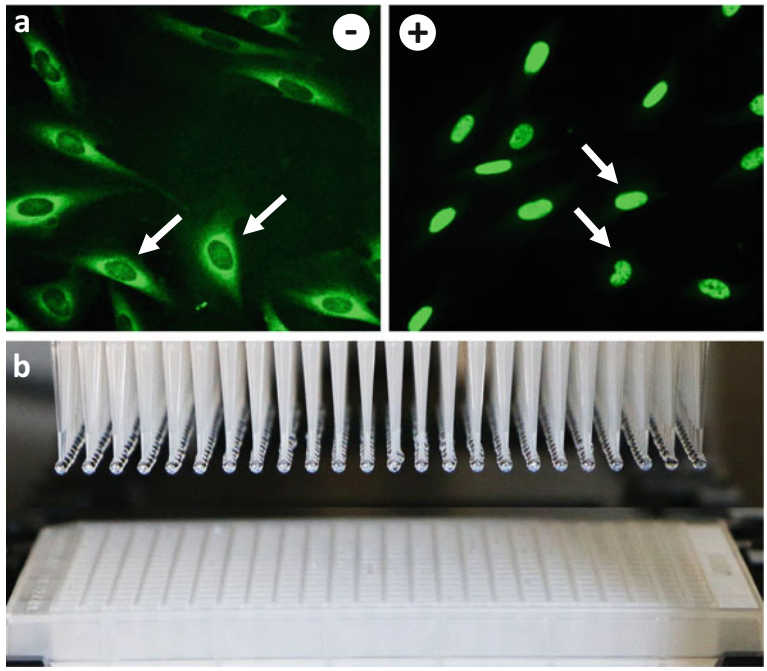
Small Molecule

A compound of natural or synthetic origin, defined by a low molecular mass of less than 1,000 daltons. Common small molecules include nucleotides in DNA, which have molecular masses of about 330 daltons; amino acids lie between 89 and 204 daltons.

Small-Molecule Screens

The process of using a library to search for small molecules that affect a biological system or experiment. Screens are often carried out using high-throughput screening (HTS), based on robotic systems and high-speed detection systems to ensure the precise transfer of reagents and accurate measurements.

The screening process comprises several steps, starting with development of a robust biological assay (Fig. 1a, b). Depending on the complexity of the biological test system, which may involve purified enzymes, cancer cells (Fig. 1a), or animal models, developing an assay for image-based screen may take up to one year. The next steps include optimizing the assay so that it can be used for moderate- to high-throughput screening in a



Small-Molecule Screens, Fig. 1 The figure summarizes the process from the development of the biological assay for high-throughput screening of compound libraries to the identification of small molecules that modulate biological functions. (**a**) *Cellular assay development:* An appropriate test is set up, with positive and negative controls such as reference drugs or an inducible cellular response. In inducible biological responses, a fluorescent protein (green color) may be sequestered in the cell cytoplasm (−); after the addition of an inducer (+), the protein may translocate to the nucleus. This example shows a transcription factor translocation to the nucleus in cancer cells, after induction and labeling by a green fluorescent protein-specific antibody. Nuclei are marked by arrows. (**b**) *Assay miniaturization:* For HTS, biological assays must be downscaled to microtiter plates containing either 1536 (48 × 32), 384 (24 × 16) or 96wells (12 × 8) per single plate and typical assay volumes ranging from 1–100 µl. Plates have a standardized footprint for robotic assay automation and are labeled by machine-readable barcodes encoding for library probes on a given test plate. Robotic grippers transport test plates from climate-controlled incubators to pipetting positions of pipetting heads with up to 1536 tips (example shows 384 disposable tips mounted at a 384-channel head pipetting liquid to a 384-well plate) or to detection systems such as microscopes or plate readers. Data are often collected and analyzed in parallel. Detection systems are either sequentially analyzing plate wells or support whole-plate imaging, for faster testing. The analysis of hits from primary screening combines different types of information, including a clustering of structurally related hits (structure-activity relationships), measurements made to detect interference with the test system, dosage-dependent interference, data regarding the purity and integrity of hits, and the frequency of hit detection in distinct tests (frequent hitters). Results are compared to public databases to assess the already known activity of structurally similar molecules

microtiter plate format with robotic automation (Fig. 1b). It needs to pass an assay acceptance test, plus a pilot screen in which a few thousand compounds are used to control the robustness of the assay in the presence of compounds and their solvent. Next comes the primary screen against the whole library of molecules. Often a counter-screen is needed to identify false positive hits which interfere with the detection reagents, coupled enzyme reactions or a cellular reporter system in an unspecific manner. Finally, primary hits are validated by testing for dosage-dependent modes of action, to exclude compounds which catalyze chemical reactions and have toxic effects. These steps depend on computational support in data documentation, the automated analysis of huge datasets, and the conversion of images into multiparameter tables. The selection of hits for further characterization and chemical optimization typically requires automatically searching

for structurally related small molecules and their known effects in public databases such as PubChem or ChEMBL, and patent databases such as Espacenet (for Europe) or USPTO (USA).

Assay development for small-molecule screens: Biological tests based on purified proteins such as enzymes, transcription factors, or scaffolding proteins provide rather simple and robust systems for screening. Often these targets play key roles in diseases due to mutations in their corresponding genes that cause hyperactivation or an impairment of their functions. Enzyme assays often use synthetic substrates whose fluorescence is altered when the enzyme is activated or inhibited. Interactions between two distinct proteins can be studied using protein-specific antibodies that only transfer a fluorescent signal if they are brought into close proximity through an interaction of the proteins. Small molecules that interfere with their interaction decrease the fluorescence, as measured with plate readers and multiwell microtiter plates. Tests with purified components permit inhibitors to be chemically optimized, altering substructures that affect their affinity and specificity for proteins and enzymes. Further tests in cells and animal models are essential to testing the bioactivity of small-molecule hits and their roles in diseases.

Cellular systems represent a next level of complexity in identifying bioactive compounds, to determine whether they can cross the cellular membrane (membrane permeability), reach proper concentrations in the cytoplasm (biostability) and act specifically on the target protein (specificity). Such tests often use cancer cells in which the endogenous target protein has become permanently activated by a mutation. The cells are genetically manipulated through the stable integration of a reporter gene into the genome whose expression depends on the activity of the protein. This reporter may encode a fluorescent protein (GFP, YFP) or an enzyme (luciferase) that produces luminescence in the presence of its substrate. Small-molecule inhibitors reduce the fluorescence or luminescence. These experiments require a counter-screen using a second, unspecific reporter cell line to identify compounds which target the reporter enzymes or fluorescent proteins themselves. Plate readers or automated microscopes are used to quantify fluorescent signals, to image the translocation of fluorescent proteins within living cells or to document cellular signal transduction. Induced pluripotent stem cells (IPSCs) have become a major source for cellular assays for screens involving heart muscle, neurons, and many other types of cells. They can be also used to generate small organ-like structures (heart or brain organoids) for screens.

Animal models for disease like nematodes or zebrafish embryos present the next level of complexity, to determine whether small molecules enrich in specific tissues and maintain sufficient concentrations over time to affect pathological processes despite the activity of secretory and metabolic clearance processes. Such screens may rely on analyses of reporter genes that encode fluorescent proteins or morphological analyses of tissues and organs.

Assay optimization: Screens of tens of thousands to a million small molecules require robust biological assays in which negative and positive controls should generate consistent readouts. Potential counteracting factors include variability between biological sample preparations, systematic variations in the measurement process, and unsystematic noise due to random influences. Biological tests need to be optimized to obtain an optimal signal-to-noise ratio and exclude an overlap due to weak signals and strong variations. Optimization affects buffer conditions for enzyme tests, enzyme and substrate concentrations, and also an optimal choice of test plates and enzyme stability. Requirements for cellular testing include an optimal density of cells and the best time window for measurements; the significant parameters for cellular responses that are to be detected with automated microscopes and image recognition software need to be identified and optimized. Assays using animal models must also be optimized for the number of animals per test and the well sizes on test plates. Furthermore, the number of repeats for compound addition and amount of compounds that are applied must be optimized for appropriate dosage using known references.

Pilot screen: After an assay passes the acceptance test, a sublibrary of a few thousand

compounds is used to test the robustness of the assay in the presence of small molecules solubilized in DMSO as a solvent. This tests for interference that arises through the physical properties of compounds (fluorescence, colored compounds) and hit rates. The pilot and primary screen may use either single point or duplicate tests and measurements at single or multiple concentrations for each compound.

Primary screen: Batches of compound plates are selected for automated processing in robotic liquid handling systems with incubators and detection systems like plate readers or automated microscopes within a single day. Depending on incubation and processing times, 10,000–50,000 tests can be finished per day. Biological responses to compound addition are analyzed on the fly to immediately identify systematic variations. Measurements of the responses are presented on a colored plate map, generally using shades of red to represent the intensity of inhibition and blue colors for activation. Each plate contains wells with compounds and negative and positive control wells to normalize the signal output for each plate over the course of the screen. This is necessary to normalize results because enzymes or reagents may become less active over time. Primary hits (activators or inhibitors) are selected and tested for dosage-dependent activity.

Primary hit validation: Depending on the complexity of the test, a counter-screen may be applied to remove primary hits that act nonspecifically. This requires controls in which signal is produced by alternative pathways or regulatory elements, rather than those specifically targeted by the test compound. Generally, selecting hits for further characterization depends on dosage-dependent effects. They are applied at a range of dosages, from those that achieve complete inhibition to low concentrations in which activity remains at 100%. Analysis reveals an IC50-value for each small-molecule inhibitor: a concentration at which the system is inhibited to 50% of its maximal activity. Selected hits are either repurchased from vendors or resynthesized to test for impurities to cause biological effects. The structures of validated small molecules are then used to search for similar molecules and select drugs for continued experiments, drawing on publicly available data on known bioactivity and patents.

Small-molecule screening libraries: Libraries contain compounds which conform to certain rules on physiochemical and structural properties that correlate with the oral availability and the success rate in drug development (Lipinski et al. 2001). These rules limit the size of drug-like compounds to a molecular mass of less than 500 daltons, the number of hydrogen bond donors and acceptors to less than 5 and 10, respectively, and the water solubility to a log P value of less than 5.

Pharmacological Relevance

The majority of drugs currently used in treatments or diagnosis are small molecules of natural or synthetic origin. This toolbox is complemented by biopharmaceuticals generated by genetically modified cells, including antibodies, peptide hormones, DNA, and RNA. In academia, small-molecule screens are mainly used in the context of chemical biology to identify bioactive small molecules for use as research tools. As dosage-dependent modulators of biological functions which can be applied and withdrawn, bioactive compounds are important complements to methods such as gene ablation or CRISPR-Cas9 gene editing.

The use of cellular screens as disease models is based on the work of Rudolf Virchow, whose theory of cellular pathology states that diseases are based on changes at the cellular level (Morrison and Weiss 2006; Virchow 1855). Paul Ehrlich established the principle of chemotherapy and launched the first small-molecule screen to search for chemicals that were toxic to pathogenic bacteria causing Syphilis (Ehrlich and Bechhold 1906). His work resulted in the development of the first effective drug against Syphilis, Salvarsan (1910–1970). The success of this systematic approach combining chemical synthesis with biological screening made this technology a standard for drug identification and development in the pharmaceutical industry.

In academia, its systematic use started with the roadmap published in 2004 by the NIH (National Institutes of Health, USA), which provided

funding for small-molecule libraries and specialized technology centers for chemical biology. European academic platforms were launched a few years later. Academic platforms and research projects are not restricted to cellular targets for modulation – the "druggable genome" which represents about 10% of human genes. They can also explore diverse other targets, such as the motor proteins that separate chromosomes during cell proliferation as anticancer targets (Overington et al. 2006; Mayer et al. 1999). There has been a steady increase in the impact of academic chemical biology projects on the validation of novel targets (first in class drugs), the development of novel technologies for screening, combinations of drug interference with genome-wide interference via RNA interference (specific degradation of gene transcripts and depletion of encoded proteins), and CRISPR-Cas9 editing (gene-specific modification or deletion of coding sequences) due to their systematic use in research.

Common Screening Technologies

Fluorescence intensity (FI): In HTS, radioactive labeling has largely given way to fluorescent labeling, thanks to progress in the development of synthetic enzyme substrates that generate fluorescence quenching and emitting side chains in one molecule, only becoming fluorescent after cleavage. Fluorescently labeled antibodies detect proteins and their interactions, exposing biochemical pathways that are activated in cells or animal models, in health or disease. Changes of the intensity of fluorescence may indicate a modulation of an enzyme's activity or the translocation of a protein in a cell. Fluorescent proteins such as GFP or YFP and others can be fused with cellular proteins to permit live-cell analyses of their locations and quantities and how they change through the activation of cellular pathways or addition of pathogens. Generally, FI detection methods provide an ideal readout for small-molecule screens (Janzen 2014).

Fluorescence polarization (FP): Exciting fixed fluorophores with polarized light results in emissions that can be detected through polarization filters. The FP technique requires a fluorescently labeled small molecule or peptide which binds to a larger binding partner such as a protein. Without the binding partner, the small-molecule label is free for dynamic rotation and emits fluorescence into all directions. Specifically binding to a protein, either as a substrate of an enzyme or as a peptide hormone to a receptor, restricts the tumbling of the labels. Applying fluorescence polarization filters during excitation and detection permits quantifying the change in rotation, which correlates to the proportion of small-molecule or protein peptide complexes that have formed. This method permits screening for small molecules which interfere with the binding of labeled ligands such as hormones or enzyme substrates. Components are only added to start the reaction, which makes the method well suited for HTS.

Fluorescence resonance energy transfer (FRET): In principle a pair of fluorophores is selected for which the first (donor) – after excitation by light –emits fluorescence at a wavelength which excites the second fluorophore (acceptor) to emit fluorescence. This only happens efficiently if the fluorophores are brought into extremely close proximity. In a FRET experiment, the two fluorophores are attached to specific proteins using, for example, antibodies which are each linked with either the donor- or acceptor-fluorophore. If two proteins bind to each other, in an in vitro or cellular assay, the energy transfer occurs after the addition of antibodies and this results in generation of a measurable fluorescent signal at the site of interaction.

Time-resolved fluorescence (TRF): Related to FRET screens, this method uses a long-wavelength donor fluorophore with a long decay time (μsec). The purpose is to avoid interference by fluorescent screening molecules, whose fluorescence generally decays within nanoseconds. Introducing a time gap to measure the signal from the second fluorophore eliminates this type of interference. This technique is widely used in very high-throughput screens of biochemical and cellular assays.

Luminescence: Isolating enzymes which use small-molecule substrates and oxygen to produce

light (bioluminescence) from a range of organisms has provided key tools to study cellular gene expression. These methods combine regulatory sequences with genes encoding these enzymes to create reporter genes. An example of their use is to monitor the binding of transcription factors to regulatory DNA elements. Permanently activated oncogenes in cancer cells, for example, can be constructed which generate luminescence if their activation is not inhibited by small molecules. In contrast to the expression of fluorescent proteins, enzymes strongly amplify transcription factor–mediated activation and thus provide a more sensitive detection of oncogenic activities. Other luminescence methods couple enzyme activity to protein interactions by fusing the enzyme and luminescence substrate to different proteins. Luminescence requires close proximity, so only sites of interaction will produce a signal. Luciferase reporter systems are also widely used in HTS.

Alpha screen: This technology delivers a chemiluminescent quantification of interactions. Two distinct proteins or interacting factors are coupled to donor or acceptor beads. The surface of each bead class is constructed to specifically bind only one type of factor. If the factors interact, this puts the beads in close proximity, triggering a laser-induced release of reactive oxygen from donor beads to acceptor beads and a chemiluminescent reaction. If small molecules inhibit binding, the beads are not coupled and the reactive oxygen becomes neutralized before reaching the acceptors. This method delivers a high number of false positive compounds which interfere with the release of reactive oxygen, which strip coupled factors from beads or interfere directly with the chemical luminescence reaction. Due to its high sensitivity, it is used, for example, to quantify levels of second messengers such as cAMP in cellular assays set up to detect G-protein-coupled receptor signaling.

Surface plasmon resonance: A protein is immobilized on a detection surface in a microfluidic chamber and a ligand such as a second protein or a small-molecule compound is added. Their binding and stability can be analyzed by measuring increases and the persistence of reflected light from the detection surface. This method permits a determination of binding constants for affinity and stability, and to detect irreversible binding or denaturation of proteins.

Capillary electrophoresis (microfluidic chips): This technology is based on hand-sized chips with up to 12 microfluidic channels and sippers which aspirate simultaneously a few nanolitres of 12 enzyme test reactions. They are capable of separating enzyme substrates and products by electrophoresis in high throughput that differ in charge – for example, after phosphorylation by kinases (which increases the negative charge of the product) or dephosphorylation by phosphatases. The enzymatic incubation is stopped after 50% of peptide substrate has been converted to product, resulting in two bands of equal amount after separation, if no inhibitor is present. The substrate is labeled by a fluorophore. This permits laser-mediated excitation and detection with a charge-coupled device camera (CCD). Applying an inhibitor decreases the fluorescence of the product band. The system permits profiling about 20.000 reactions per day and provides high-quality data, because detection is not affected by fluorescence quenching or unspecific physical properties of compounds.

Flow cytometry: This method is used to analyze cells in suspension, not adhered to a surface. Cells are marked by fluorescent dyes or antibodies of biological relevance, indicating, for example, their proliferative state or developmental stage, then excited by a laser. This permits very sensitive detection and readouts. Tens of thousands of cells in one test well can be sorted by size and fluorescence intensity, which means, for example, that a cancer marker can be detected on different cell types in a single experiment.

High-content screening and machine learning: Automated microscopes with 2D or 3D functions through confocal imaging are currently widely used to profile morphological alterations of cells derived, for instance, from patients. This broad data collection is termed high-content screening, and it is currently being used in conjunction with iPSC technologies to study 3D organoids and other structures. Applying drugs to organoids for colon cancer, for example, has been shown to

produce results that more closely resemble those of clinical trials than other current methods.

High-content screening requires the automated, software-guided identification of cellular objects such as nuclei, filaments, and membrane compartments. These structures are automatically recognized and converted to parameters representing size, fluorescence intensity, etc. Sometimes, small changes in specific parameters may provide a more significant readout for cellular responses than larger ones which may be more obvious in a visual examination. Therefore, machine learning is combined with a human visual sorting of experimental outcomes to develop computer-guided, positive, and negative correlations and filter a training set of data. Afterwards this artificial intelligence can be used to analyze image-based HTS data.

Impedance (label free): This method makes use of microtiter plates with golden electrodes at the bottom. A low electric current is applied to analyze changes in the electric resistance of cells adjacent to the electrodes. The impedance may change, for example, through signaling processes which cause a release of calcium from endogenous stores, or induce a stronger attachment to electrodes. In contrast to other methods, no chemical fixation of cells and membrane permeabilization for antibody detection is required, resulting in dead cells, instead living cells can be analyzed over time without further treatment (label free).

Automated data documentation and analysis: The high-throughput screening of large small-molecule libraries requires the automated documentation and analysis of the data that is acquired. This can be achieved by adapting commercial and free open-source software. Biological and chemical data such as compound identifiers and structure information must be linked in the documentation. Scripts that are appropriate to the screening technology must be written to filter and analyze hits and to set curves to fit kinetic or end-point data collections. A conversion of measurements to colored heat maps reflecting biological activities permits a rapid detection and correction of systematic errors. Researching structural similarities and known bioactivities in public databases is essential in designing follow-up tests and selecting candidates for further study. Small-molecule screening represents a dynamic marriage of technologies with biological, chemical, and computational expertise in an inherently interdisciplinary enterprise that holds great promise for the future identification and development of potent new drugs.

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Smooth Muscle Tone Regulation

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Synonyms

Regulation of smooth muscle contractility

Definition

The following organs contain as major functional part smooth muscle layers: arterial and venous

vessels; lung and bronchia; oesophagus, stomach, and small and large intestine; urinary tract and bladder; and uterus. Hormones, locally released transmitters and shear stress or pressure regulate the tonus of these organs. Each organ has a slightly different regulation of its contractility, but the basis for this regulation, i.e. the intracellular signaling pathways, is very similar or identical. This article will focus on major findings that may be identical in all smooth muscles.

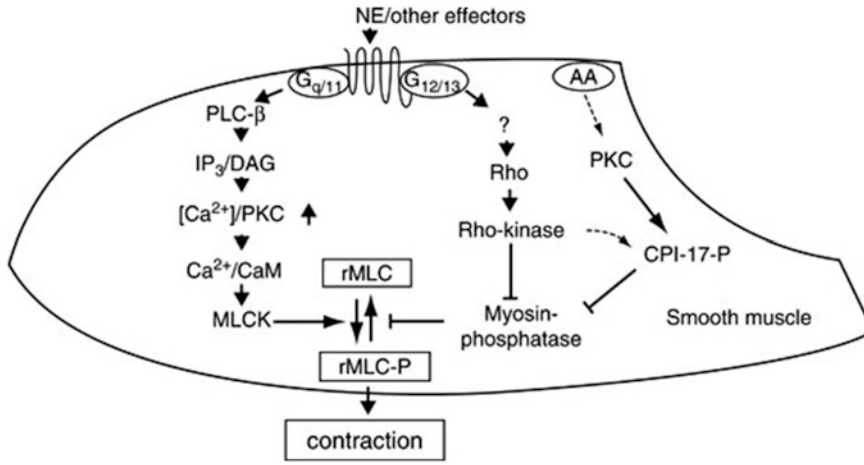
Basic Mechanisms

Key mechanism of smooth muscle tone regulation is the phosphorylation of Ser-19 of the regulatory myosin light chain II (rMLC) (Pfitzer 2001). Phosphorylation and dephosphorylation are catalysed by myosin light chain kinase (MLCK) and the type 1 myosin phosphatase (MLCP), respectively. Calcium-dependent and calcium-independent signal pathways regulate the activity of both enzymes and thereby the phosphorylation status of rMLC. An increase in the cytosolic calcium concentration leads to phosphorylation of the rMLC and contraction within 4 s. The correlation between percent phosphorylated rMLC and developed force is quite variable. Maximal force can be attained at 0.2–0.3 mol phosphate per mol rMLC. Phosphorylation can decline during maintenance of tension suggesting that even dephosphorylated cross-bridges can contribute to force maintenance.

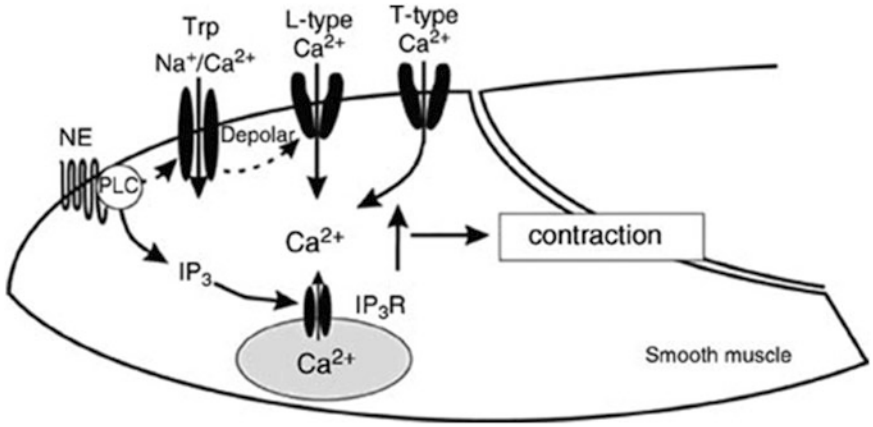
Calcium-dependent regulation involves the calcium-calmodulin complex that activates smooth muscle MLCK, a monomer of approximately 135 kDa. Dephosphorylation is initiated by MLCP. MLCP is a complex of three proteins: a 110–130 kDa myosin phosphatase targeting and regulatory subunit (MYPT1), a 37 kDa catalytic subunit (PP-1C) and a 20 kDa subunit of unknown function. In most cases, calcium-independent regulation of smooth muscle tone is achieved by inhibition of MLCP activity at constant calcium level inducing an increase in phospho-rMLC and contraction (Fig. 1).

Calcium-Dependent Contraction

Different agonists such as norepinephrine, acetylcholine or angiotensin II activate smooth muscle contraction by binding to a heptahelical receptor, i.e. α-adrenergic, muscarinergic or AT-1 receptors, followed by an increase in cytosolic calcium (Figs. 1 and 2). The activation of the trimeric G proteins G_q or G_{11} increases the activity of phospholipase Cβ (PLC) generating inositol trisphosphate (IP_3) and diacylglycerol (DAG) and other fatty acid-derived compounds. Classical



Smooth Muscle Tone Regulation, Fig. 1 Mechanisms leading to agonist-stimulated calcium-dependent and calcium-independent contraction of smooth muscle. NE, norepinephrine. See text for the other abbreviations



Smooth Muscle Tone Regulation, Fig. 2 Membrane mechanisms leading to an increase in cytosolic calcium concentration. Depolar, depolarization of the membrane; see text for abbreviations

findings suggested that IP_3 stimulates calcium release from intracellular stores that binds to calmodulin and activates MLCK. This simple scheme is not in line with the fact that a block of the L-type Ca^{2+} channel inhibits contraction. The importance of the L-type calcium channel is further supported by genetic deletion of the corresponding $Ca_v1.2$ gene. Mice lacking the smooth muscle Cav1.2 channel have severe difficulties to contract intestinal and other smooth muscle. It is therefore likely that activation of a heptahelical receptor leads to depolarization of the membrane and activation of the L-type calcium channels (Fig. 2). Possible candidates are TRP channels (most likely TRPC6) activated either by DAG or by interaction with empty IP_3 stores. The inflowing cations may depolarize the membrane to potentials that activate T- and thereafter L-type calcium channels or directly L-type channels. A second channel depolarizing the membrane is the calcium-activated chloride channel present in many smooth muscle cells. Calcium released from IP_3 stores or flowing in through TRP channels could activate this chloride channel and depolarize thereby the membrane. The L-type calcium channel provides calcium to trigger calcium release from ryanodine receptor-controlled calcium stores and for refilling various intracellular calcium stores (Jaggar et al. 2000). The mechanism behind pressure- or shear stress-induced contraction is unsolved but may again involve TRP channels and mechanosensitive cation channel Piezo1. Piezo1 is mainly expressed in endothelial cells. Shear stress activates the endothelial Piezo1 and contributes thereby to endothelial-dependent hyperpolarization (EDH) and relaxation of arterial resistance vessels (Douguet et al. 2019).

Calcium-Independent Contraction

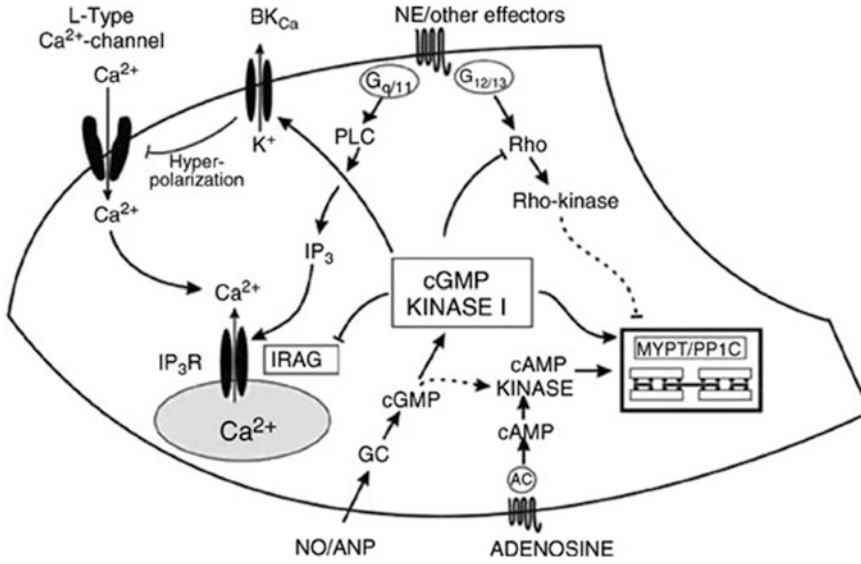
Agonist-activated receptors can induce contraction at a constant intracellular calcium concentration (Somlyo and Somlyo 2000), if the receptor activates the G proteins G_{12} or G_{13} (Fig. 1). Activation of these G proteins recruits the monomeric GTPase Rho to the membrane, where Rho exchanges GDP against GTP and activates Rho kinase. By a still unsolved cascade eventually involving ZIP kinase, the MYPT1 subunit of MLCP is phosphorylated at Thr-697 and Thr-854 (rat MYPT1) which reaction inhibits MLCP activity. Since the activity of MLCK is not affected by this cascade, rMLC is phosphorylated to a higher level. Phosphorylation and inhibition of MLCP activity are only observed, if a central exon of MYPT1 is present. These results could explain the old finding that certain agonists induce calcium sensitization of the contractile machinery in most but not all smooth muscles. MLCP activity is also affected by a smooth muscle-specific

inhibitor protein of PP-1C, named CPI-17. Protein kinase C (PKC) phosphorylates CPI-17, which becomes then a high affinity inhibitor of the catalytic subunit of MLCP. The nature of the PKC subtype is not clear. It is possible that it is one of the atypical PKC enzymes that is activated directly (?) by arachidonic acid (AA). Rho kinase which phosphorylated CPI-17 in vitro apparently may affect directly in vivo the phosphorylation status of CPI-17 (Dimopoulos et al. 2007). Arachidonic acid inhibits dephosphorylation of MLCs, i.e. by a second mechanism by dissociating the MLCP holoenzyme.

Relaxation of Smooth Muscle

The major relaxing transmitters are those that elevate the cAMP or cGMP concentration (Fig. 3). Adenosine stimulates the adenosine receptor A_2, increases cAMP and activates thereby the activity of cAMP kinase. The next step is not clear, but evidence has been accumulated that cAMP kinase decreases the calcium sensitivity of the contractile machinery. In vitro, cAMP kinase phosphorylated MLCK and decreased thereby the affinity of MLCK for calcium-calmodulin. However, this regulation does not occur in intact smooth muscle. Possible other substrate candidates for cAMP kinase are the heat-stable protein HSP 20, a heat stable protein of 20 kDa that is phosphorylated by cGMP kinase. It has been postulated that phospho-HSP 20 interferes with the interaction between actin and myosin allowing thereby smooth muscle relaxation without dephosphorylation of the rMLC. Rho A and MLCP are phosphorylated also by cGMP kinase I (Fig. 3).

A major relaxing factor is NO, a signal molecule synthetized by three different NO synthases (NOS). NO synthetized in the endothelial layer of the vessels diffuses into the smooth muscle layer, where NO activates soluble guanylate cyclase (GC) and generates high concentrations of cGMP. In non-vascular systems such as the intestinal smooth muscle, NO is released from non-adrenergic, non-cholinergic neurons. An alternative pathway for the production of cGMP is the stimulation of particulate GC by the natriuretic peptides ANF, BNF and CNF. ANF and BNF are released from cardiac atrial and ventricular muscle, respectively, and lower blood pressure. These effects of the natriuretic peptides are mediated through cGMP and cGMP kinase I. NO has additional effects which



Smooth Muscle Tone Regulation, Fig. 3 Major mechanisms leading to relaxation of smooth muscle. See text for the abbreviations

are not mediated by cGMP kinase I. CNP is released from the vascular endothelial layer and exerts its relaxing effects either through activation of the particulate guanylyl cyclase B identical with the NPR-B or through the NPR-C receptor. The endothelial layer of the vessels initiates a number of diverse signaling pathways that lead to endothelium-dependent hyperpolarization (EDH) and relaxation of vascular smooth muscle (Garland and Dora 2017).

Smooth muscle contains the two cGMP kinase isozymes Iα and Iβ and a number of identified substrates (Hofmann et al. 2006). The NO/cGMP/cGMP kinase pathway interferes with the calcium-dependent and the calcium-independent contraction. A number of researchers have shown that cGMP-dependent phosphorylation of the BK_{Ca} channel increases its open probability resulting in hyperpolarization of the membrane potential and closure of voltage-dependent calcium channels. The activity of the BK_{Ca} channel is upregulated by the intracellular calcium concentration establishing a negative feedback loop. It is well established that cGMP kinase decreases the release of calcium from intracellular stores. Recently, it was found that cGMP kinase Iβ is associated with the IP_3 receptor type 1 and the 130 kDa protein IRAG. Phosphorylation of IRAG inhibited the release of calcium from IP_3-sensitive stores in COS cells. However, isozyme-specific reconstitution of cGMP kinase I-deficient mice suggested that, in murine aortic smooth muscle cells, cGMP kinase Iα and Iβ lowered norepinephrine-stimulated increases in the cytosolic calcium concentrations. This result is in line with the recent notion, which calcium-dependent contraction of smooth muscle requires membrane depolarization and calcium influx through membrane localized ion channels (see above). It is possible that the IP_3-sensitive calcium pool associated with IRAG and cGKIβ controls other smooth muscle functions such as phenotype changes and smooth muscle growth.

It was demonstrated that cGMP kinase I inhibits also smooth muscle contraction due to the calcium-insensitive pathway. A possible mechanism could be phosphorylation of Rho by cGMP kinase I. The phosphorylation site is identical with a cAMP kinase site identified in Rho from non-smooth muscle cells. It was reported that phosphorylation of Rho by cGMP kinase I prevents membrane association of Rho that is required to stimulate the GDP/GTP exchange. Alternatively, phosphorylation of telokin may interfere with the calcium sensitization of contraction. cGMP kinase Iα interacts specifically with a leucine zipper present at the C-terminus of MYPT1. Depending on the tissue, this leucine zipper is present or not. MLCP activity increased when MYPT1 is phosphorylated by cGMP kinase Iα at Ser-696.

Pharmacological Intervention

A large number of drugs interfere with the smooth muscle contraction. These compounds lower blood pressure and are referred to as antihypertensive. In this section, only those compounds will be mentioned that have a direct effect on smooth muscle tone. Phenylephrine is an agonist on most smooth muscles and activates α_1 adrenoceptors. Carbachol is an agonist on some smooth muscles and activates contraction through muscarinic receptors. Blockers of the α_1-adrenoceptors such as prazosin and urapidil are competitive inhibitors of the α_1-receptor in vascular and bladder smooth muscle. Phenoxybenzamine is an irreversible blocker of α_1 receptors, and phentolamine blocks α_1 and α_2 receptors. Ca^{2+} channel blockers such as the dihydropyridines, phenylalkylamines and benzothiazepines lower smooth muscle tone by blocking the L-type calcium channel.

Nitrates (glyceryl trinitrate, isosorbide dinitrate, pentaerythritol tetranitrate, molsidomine, sodium nitroprusside) that generate NO increase cGMP concentrations and lower smooth muscle tone by activation of cGMP kinase I. Nitrates relax in vivo mainly the capacitive part of the circulation system, i.e. the venous part. Sildenafil, a specific inhibitor of phosphodiesterase 5, a cGMP hydrolysing enzyme, increases cGMP in the corpus cavernosum and lowers together with nitrates the blood pressure by the combined effect on cGMP level. An additional potentially important drug family is compounds that stimulate the soluble guanylyl cyclase independent of NO (Evgenov

et al. 2006) and lower elevated blood pressure. The inhibitor of Rho-kinase, Y-27632, lowers elevated blood pressure in hypertensive animals, without affecting significantly the blood pressure of non-hypertensive animals (Uehata et al. 1997).

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S-Nitrosation

▶ Protein S-Nitrosylation

Sodium- and Potassium-Activated Adenosine 5′-Triphosphatase (EC 7.2.2.13)

▶ Na^+/K^+-ATPase

Sodium Calcium Exchanger

▶ Na^+/Ca^{2+} Exchangers

Sodium Pump

▶ Na^+/K^+-ATPase

Sodium/Proton Antiporter

▶ Na^+/H^+ Exchangers

Sodium/Proton Exchanger

▶ Na^+/H^+ Exchangers

Soluble Epoxide Hydrolase

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Synonyms

EPHX2; Epoxide hydrolase 2; sEH

Definition

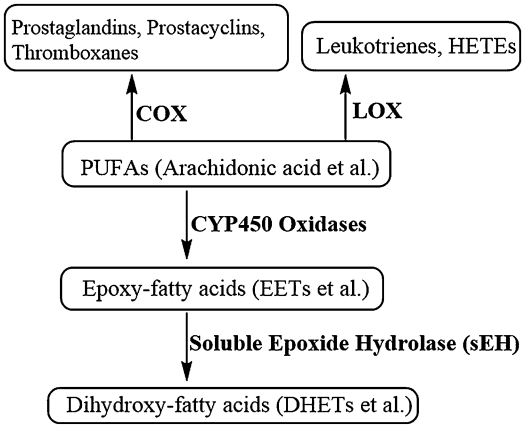
The epoxide hydrolase (EH) family of enzymes is responsible for converting the epoxide residue of various endogenous and exogenous compounds to the corresponding vicinal diols (Morisseau and ammock 2005). Soluble epoxide hydrolase (sEH) is an enzyme in the α-/β-hydrolase fold protein family, primarily found in the cytosolic and peroxisomal fractions of cells (Chiamvimonvat et al.

2007). It is encoded by the EPHX2 gene. It is a bifunctional homodimer that has EH activity at the C-terminal domain and phosphatase activity at the N-terminal domain (He et al. 2016). sEH is abundantly expressed in the liver, but it is also present in most organs including the kidney, brain, smooth muscle, intestines, and lungs (Morisseau and Hammock 2005). Its substrate selectivity is largely restricted to noncyclic 1,2-disubstituted epoxides, and hence, unlike the microsomal epoxide hydrolase (mEH), it is generally not involved in the metabolism or detoxification of xenobiotics (Morisseau and Hammock 2005). On the other hand, polyunsaturated fatty acid (PUFA)-derived bioactive lipid mediators are usually good sEH substrates and are subject to hydrolysis. These epoxy fatty acids (EpFAs) have demonstrated numerous tissue healing and organ-protective effects. sEH inhibitors (sEHI) help stabilize endogenous levels of EpFAs, thus enhancing their bioavailability and facilitating their biological action. Hence, targeting sEH with novel and potent small-molecule inhibitors is a pharmacological tool employed to capitalize on the beneficial effects of EpFAs and potentially treat several disease conditions (Morisseau and Hammock 2005). Alternative approaches involve (i) administration of EpFAs themselves, (ii) synthesis and application of EpFA mimics, and (iii) stimulation of EpFA biosynthesis.

Basic Characteristics

Metabolism of PUFAs

Arachidonic acid (20:4, ω-6) is a PUFA derived from the phospholipids of cell membranes and is metabolized by three main enzymatic pathways (Fig. 1) (Imig and Hammock 2009). The cyclooxygenase (COX) and lipoxygenase (LOX) routes generate largely pro-inflammatory mediators and are the target of several pharmaceuticals currently available on the market (e.g., nonsteroidal anti-inflammatory drugs a.k.a. NSAIDs and selective COX-2 inhibitors a.k.a. COXIBs). The third pathway primarily involves cytochrome P450-mediated epoxidation to epoxy metabolites known as epoxyeicosatrienoic acids (EETs). Other ω-3



Soluble Epoxide Hydrolase, Fig. 1 Schematic depicting major pathways of polyunsaturated fatty acid (PUFA) metabolism

PUFAs such as docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5) are similarly metabolized, and the CYP450 branch generates epoxydocosapentaenoic acids (EDPs) and epoxyeicosatetraenoic acids (EEQs), respectively (He et al. 2016). EETs and other EpFAs are mono-epoxides that are chemical mediators and act in both autocrine and paracrine manners. However, the chemically stable EpFAs have transient metabolic half-lives due to rapid sEH-mediated degradation. Recently, epoxides of omega-3-derived endocannabinoids with anti-inflammatory, anti-tumorigenic, and analgesic function have been reported, suggesting a still broader role of sEH in regulatory biology (Das et al. 2019).

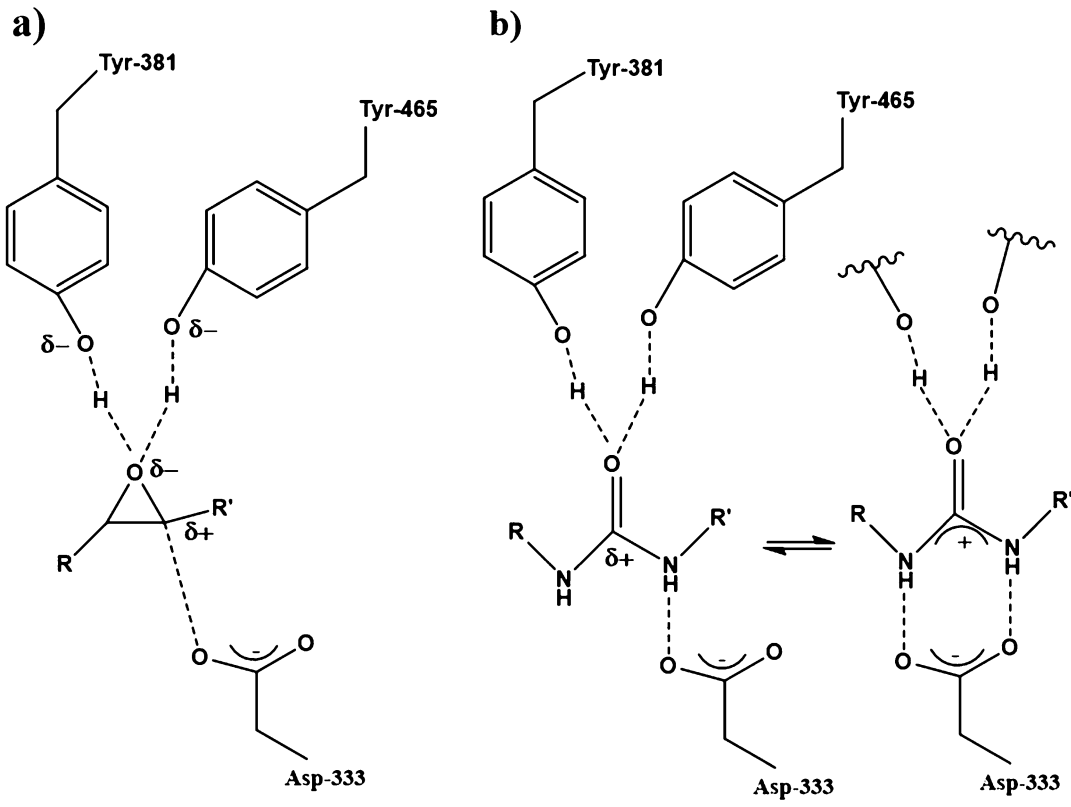
Hydrolysis of EpFAs

sEH hydrolyzes EETs and other EpFAs by an exothermic, two-step base-catalyzed mechanism (Morisseau and Hammock 2005). It involves the formation of a covalent hydroxyl-alkyl-enzyme ester intermediate via a catalytic aspartic acid, followed by the attack of water on the enzyme ester carbon that releases the 1,2-diol product. Within the hydrophobic active site, two tyrosine residues hydrogen bond to the epoxide oxygen, polarizing it. A nucleophilic aspartate acid conducts a backside attack on the epoxide, forming the covalent intermediate, while an

additional aspartate residue acts as orienting and activating acid for the histidine general base. This activates a water molecule which conducts the described hydrolysis step. sEH metabolism of EpFAs is regioselective and stereoselective (Chiamvimonvat et al. 2007). For example, the EET regioisomer with the epoxide furthest from the carboxylic acid end (14,15-EET) is hydrolyzed the fastest, while the one closest is hydrolyzed most slowly (5,6-EET).

Mechanism of sEH Inhibition

Enzyme inhibitors acting to mimic reaction intermediates or transition states have been found as potent inhibitors in many α-/β-hydrolase fold enzymes (Morisseau and Hammock 2005). Based on the catalytic mechanism of sEH, the transition states of epoxide ring opening are effectively mimicked by the urea functional group (Fig. 2). The C=O moiety accepts hydrogen bonds from the two tyrosine residues and hence acts as a surrogate for the epoxide oxygen. One mechanism suggests that one of the urea NH groups donates a hydrogen bond to the nucleophilic aspartate, mimicking the electrophilic center (Fig. 2). Alternatively, both NH groups donate hydrogen bonds to the aspartate, stabilizing a partial salt bridge with the enzyme, and thus mimic the interaction between the enzyme and substrate during the transition states (Fig. 2). Accordingly, a series of small-molecule 1,3-disubstituted ureas as well as amides, carbamates, and heterocycles have been developed which act as slow, tight-binding, competitive, and highly potent (low nanomolar to picomolar IC_{50}) inhibitors of sEH (Morisseau and Hammock 2005). The functional groups attached to the primary urea pharmacophore are generally hydrophobic to allow effective interaction with



Soluble Epoxide Hydrolase, Fig. 2 (**a**) Transition state of epoxide ring opening within the sEH active site. (**b**) Two possible binding modes of 1,3-disubstituted urea inhibitors mimicking multiple transition states and reaction intermediates along the reaction coordinate

the sEH active site, with a smaller group on one side and a typically larger group on the other. A polar moiety (e.g., ether, ester, alcohol, or ketone) added to the side with the larger group can act as a secondary pharmacophore and improve water solubility, pharmacokinetic parameters, and drug formulation properties, without lowering its potency (Imig and Hammock 2009).

Biological Action of EETs and EpFAs

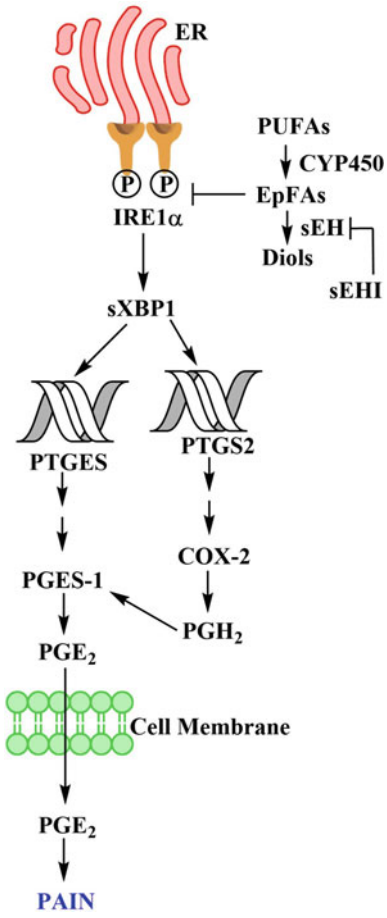
The inhibition of sEH has yielded therapeutic potential in models for various inflammatory, hypertensive, metabolic, fibrotic, and neurodegenerative diseases. The physiological effects of sEH inhibitors stem from the upregulation and subsequent action of EpFAs. The lack of a defined receptor for EETs and other EpFAs makes the assessment of the specific mode of action more challenging. Nevertheless, EpFAs are known to exert several biological effects and are hypothesized to act through multiple pathways.

Inhibition of sEH attenuates and resolves chronic inflammation through multiple pathways (Imig and Hammock 2009). Certain EETs such as the 11,12-regioisomer prevent nuclear translation of NF-κB and thereby block transcription of genes that encode pro-inflammatory enzymes (e.g., iNOS, LOX-5, COX-2, PGE2 synthase). 11,12-EET reduces IKK activity, which decreases phosphorylation and degradation of IκB, allowing it to sequester NF-κB in the cytosol. Other pathways include STAT3 tyrosine-705 phosphorylation by 14,15-EET, PPAR-alpha and PPAR-gamma agonism, inhibition of cytokine-induced VCAM-1, reduced TNFα secretion and, as discussed later, the endoplasmic reticulum (ER) stress response.

sEH inhibition has also been associated with smooth muscle relaxation and vasodilatory function (Imig and Hammock 2009). EETs, specifically the 11,12- and 14,15-regioisomers, are endothelium-derived hyperpolarizing factors (EDHFs). They stimulate calcium-activated potassium channels (K_{Ca}^{+}), triggering an efflux of K^{+} from vascular smooth muscle cells. This results in membrane hyperpolarization and subsequent dilation of blood vessels. cAMP activation of protein kinase A (PKA) and ADP ribosylation of the alpha subunit of G_s are mechanisms by which EETs have been demonstrated to activate K_{Ca}^{+} channels and promote vascular relaxation (Imig and Hammock 2009).

Disruptions in endoplasmic reticulum (ER) homeostasis caused by reactive oxygen species (ROS), high glucose, or xenobiotic perturbations result in ER stress. The ability of EpFAs to attenuate ER stress and downregulate the triggered unfolded protein response (UPR) pathways has been shown to underlie the amelioration of multiple metabolic disorders including fibrosis, inflammatory, and neuropathic pain (Fig. 3) (Inceoglu et al. 2017). sEHI modulation of the PERK/eif2α branch of the UPR enhances systemic insulin sensitivity and improves glucose tolerance (Inceoglu et al. 2017). It is also considered to play a role in ensuring proper pancreatic exocrine functionality and preventing pancreatitis. Mitigation of the IRE1α and ATF6 UPR pathways by sEH inhibition decreases the TGF-β-induced myofibroblastic differentiation of lung fibroblasts and helps prevent the development of lung fibrosis. The inhibition of sEH has also exhibited a promising analgesic action toward diabetic neuropathy and other types of chronic pain. One proposed mechanism suggests downregulation of the three UPR arms in peripheral nerves, which reduces predisposition to aberrant axonal firing and decreases apoptotic signaling and oxidative stress (Inceoglu et al. 2017).

The inhibition of sEH and resulting elevated EpFA levels in the brain is considered to increase brain-derived neurotrophic factor (BDNF) levels in the prefrontal cortex and hippocampus (Hashimoto 2019). BDNF binding to tropomyosin receptor kinase B (TrkB) and enhanced BDNF-TrkB signaling promotes synaptogenesis and corrects dopaminergic dysfunction, leading to an improvement in depressive and schizophrenic symptoms. EpFAs also seem to play a role in preventing phosphorylation of α-synuclein and sequentially reducing the aggregation of α-synuclein in multiple brain regions (Hashimoto 2019). By blocking the deposition of phosphorylated α-synuclein aggregates (a.k.a. Lewy bodies), the loss of dopaminergic neurons from the



Soluble Epoxide Hydrolase, Fig. 3 Epoxy fatty acids (EpFAs) preserved by sEH inhibitors (sEHI) stabilize mitochondria against disruption, reduce reactive oxygen species (ROS), and shift the endoplasmic reticulum (ER) stress response away from initiating cell damage and inflammation and back toward maintaining homeostasis (Inceoglu et al. 2017). Downstream from mitochondrial stabilization and reduction in ROS, EpFAs alter ER stress-induced unfolded protein response (UPR) pathways. For one pathway acting through IRE1α (shown above), sEHI reduce protein and message for COX-2 (Schmelzer et al. 2005) and PGES-1 (Chopra et al. 2019), thus dramatically reducing PGE_2 and other inflammatory and pain-initiating eicosanoids

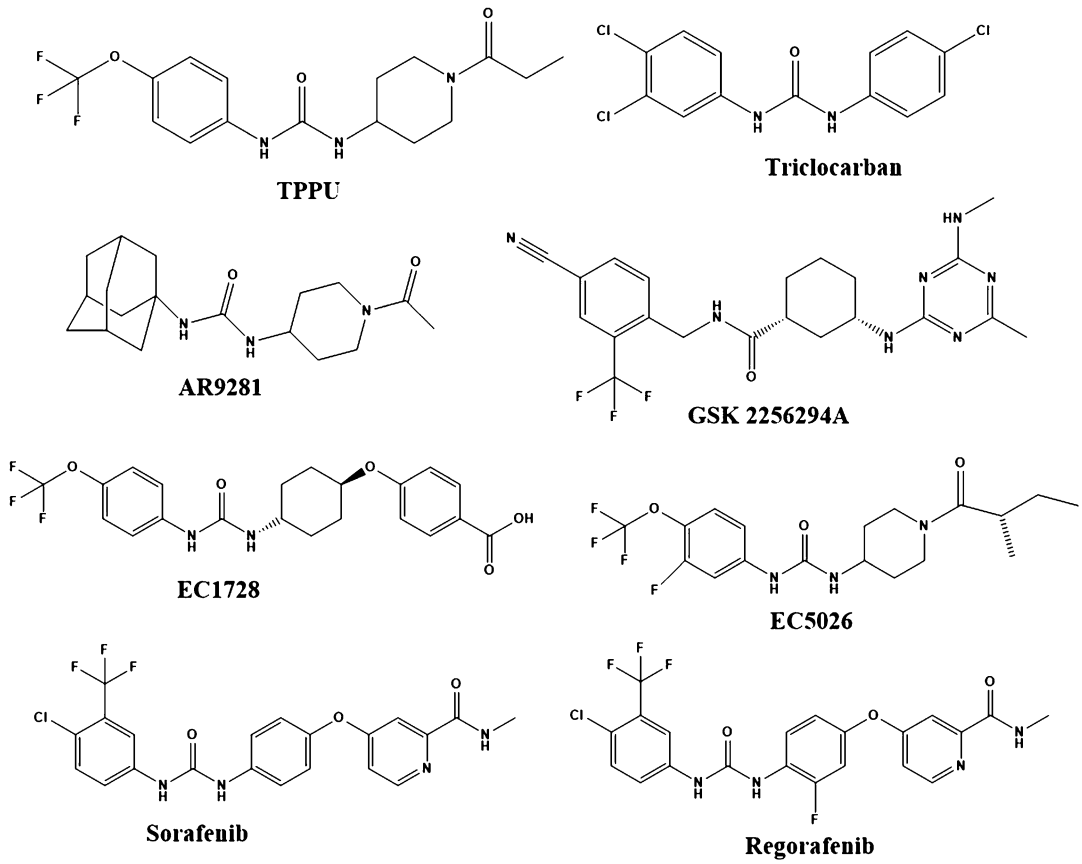
substantia nigra is potentially mitigated, and the pathogenesis of Parkinson's disease and dementia with Lewy bodies (DLB) may be controlled.

For pain as an endpoint in animal studies, the inhibition of sEH forgoes the common adverse side effects of NSAIDs and COXIBs (e.g., GI ulceration, cardiovascular issues) (Wagner et al. 2017). Additionally, it displays none of the reward-seeking addictive potential associated with the use of opioid narcotics (Wagner et al. 2017). EETs can be angiogenic at very high doses, and sEHI upregulation of EETs is considered to slightly promote tumor growth. This phenomenon has been attributed to downstream COX metabolites of EETs (Rand et al. 2017). Combining sEH inhibitors and COX inhibitors has been shown to have significant anti-angiogenic effects and blunt tumor growth, as has the use of sEHI in animals with an enhanced omega-3 and depleted omega-6 diet.

Drugs

Representative sEH Inhibitors

TPPU (i.e., UC1770) is extensively used in animal disease models because it is orally bioavailable and has favorable pharmacokinetic characteristics in addition to high potency (Fig. 4). Currently, no clinical drug on the market is used as an sEH inhibitor. However, antineoplastic agents sorafenib and regorafenib, inhibitors of vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and Raf family kinases are powerful sEH inhibitors (Fig. 4) (Liu 2019). This activity likely reduces severe toxicity associated with the use of protein kinase inhibitors. Triclocarban, a commonly used antibacterial, has also displayed sEH inhibitory potential (Fig. 4) (Liu 2019). These compounds contain the ideal disubstituted urea substructure which indicates the diversity of structures that can inhibit the sEH. Several pharmaceutical companies have been or are in the process of discovering and developing potent sEH inhibitors in order to treat several clinical indications. These include Arête Therapeutics, Boehringer Ingelheim, Taisho, Merck, GlaxoSmithKline (GSK), and EicOsis. One compound, AR9281 (i.e., UC1153 or APAU) from Arête, was designed to target moderate hypertension and impaired glucose tolerance (Fig. 4). It completed Phase I and Phase IIA clinical trials. AR9281 was well tolerated for both a single oral dose and multiple doses.



Soluble Epoxide Hydrolase, Fig. 4 Chemical structures of representative sEH inhibitors

However, as expected from the adamantane in the structure, AR9281 had poor pharmacokinetic and pharmacodynamic parameters suggesting at least a twice- or thrice-daily dosing regimen for effective sEH inhibition (Liu 2019). Not only was the inhibition of the human enzyme weak, but the target occupancy was also poor. Despite no safety concerns in clinical trials, AR9281 failed to show efficacy in early-stage hypertension indicating hypertension might not be a suitable primary clinical indication for sEHI and AR9281 was not appropriate for sustained inhibition of the target sEH. Another inhibitor, GSK2256294A from GSK, was targeted to treat chronic pulmonary obstructive disease (CPOD) and completed Phase I clinical trials (Fig. 4). It has shown no adverse effects and a dose-dependent increase in plasma drug levels and sEH inhibition (Liu 2019). EicOsis designs pain therapeutics, and the compound EC1728 (i.e., *t*-TUCB) has been shown to successfully treat equine laminitis as well as inflammatory pain in dogs and cats (Fig. 4). Currently, EC1728 is pending an FDA review to begin Phase 1 trials in horses in 2020. The inhibitor EC5026 has been selected for treating diabetic neuropathy and other forms of neuropathic pain in patients while promoting opioid-sparing effects (Fig. 4). It has displayed considerable efficacy in preclinical studies as well as ideal pharmacokinetic properties and stability. The IND has been filled, and it entered Phase 1 clinical trials in December of 2019.

So far, sEHI have generally demonstrated few to no adverse side effects even at relatively high doses, and they have an excellent therapeutic

index. However, the broad range of biological effects observed with the use of sEHI, like other drugs acting on the arachidonate cascade, suggest they should be utilized with caution.

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Solute Carrier 9 Family

▶ Na^+/H^+ Exchangers

Somatostatin

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Definition

Somatostatin, also known as somatotropin-release inhibitor factor (SRIF), is a cyclic neuroendocrine peptide that was first isolated and identified as a hypothalamic peptide that inhibited growth hormone (GH) secretion from anterior pituitary cells (Brazeau et al. 1973). SRIF is expressed in the central nervous system (CNS) and in peripheral tissues and exerts potent inhibitory actions on neuronal excitability, hormone secretion, and exocrine secretion. SRIF also negatively affects smooth muscle cell contraction and cell proliferation. Its physiological functions are mediated by five G protein-coupled receptors (GPCRs) called somatostatin receptors ($SST_{1–5}$).

Basic Characteristics

Somatostatin and Somatostatin-Related Peptides

SRIF exists in two main isoforms: the tetradecapeptide SRIF-14 and the 28-amino-acid peptide SRIF-28. The two isoforms are generated from the same precursor through post-translational processing at a distinct cleavage site and are expressed at variable amounts in the same tissues. The SRIF-14 primary structure and cleavage sites generating the two isoforms have been highly conserved

during evolution in vertebrates (Conlon et al. 1997). In humans, the somatostatin precursor (pre-proSRIF) is encoded by the SS1 gene, which is localized on chromosome 3q27.3. Two additional products of the SRIF precursor have been isolated in mammals. Neuronostatin is a 13-amino-acid noncyclic amidated peptide whose sequence immediately follows the signal peptide (Samson et al. 2008; Yosten et al. 2015). A second peptide expressed in enteric neurons comprises the N-terminal 13 amino acids of SRIF-28 (Ensinck et al. 2002, 2003). In 1996, a neuropeptide showing high similarity to SRIF was identified and termed cortistatin (CST) (de Lecea et al. 1996). CST is encoded by the *CORT* gene (Liu et al. 2010). Cleavage of preproCST gives rise to two mature products, CST-17 and CST-29, in humans. CST is also structurally and functionally related to the urotensin II peptide family. The CST precursor is mainly expressed in the cerebral cortex and hippocampus. It is also expressed in the periphery but at lower levels than SRIF.

Tissue Distribution

In the CNS, SRIF has been localized in the mediobasal hypothalamus and median eminence, amygdala, preoptic area, hippocampus, striatum, cerebral cortex, and the brainstem (Johansson et al. 1984). SRIFergic neurons can be distinguished in hypophysiotropic neurons, long-projecting GABAergic neurons, and GABAergic interneurons (Viollet et al. 2008; Urban-Ciecko and Barth 2016). In the periphery, SRIF is expressed in secretory cells in gastrointestinal mucosa, δ-cells in pancreatic islets, and a subpopulation of C cells within the thyroid gland. SRIF is also produced by inflammatory and immune cells (Patel 1999) and has been localized in human epidermis (Merkel and dendritic cells) (Vockel et al. 2010, 2011). Finally, SRIF is believed to function as a neurotransmitter, neuromodulator, or trophic factor in the retina (Cervia et al. 2008). Plasma levels of SRIF are very low because of rapid degradation by ubiquitous peptidases.

Physiology

SRIF is a neurotransmitter, neuromodulator, and paracrine factor acting in the same tissues where it is expressed. Hypothalamic SRIF is the main inhibitory factor involved in the control of GH secretion and reduces the release of prolactin and thyroid-stimulating hormone (TSH) (Müller et al. 1999). In the brain, SRIF actions are mediated by presynaptic or postsynaptic mechanisms. SRIF modulates neuronal excitability, and in the hippocampus, cortex, and hypothalamus, it causes presynaptic inhibition of excitatory neurotransmission (Peineau et al. 2003). Finally, it is co-released with GABA from axonal terminals in different brain areas (Olias et al. 2004). SRIF has a role in cognitive functions, learning, memory, control of locomotor activity, food intake, nociception, and autonomic functions. SRIF is highly expressed in regions associated with seizures and has been suggested to act as an endogenous antiepilectic (Stengel et al. 2015). In the gastrointestinal tract, SRIF exerts a generalized inhibitory effect on release of gut hormones and exocrine secretion (gastric acid, digestive enzymes, bile, and colonic fluid). SRIF also negatively affects gallbladder contraction, small intestinal segmentation, and gastric emptying. In pancreatic islets, release of SRIF inhibits secretion of insulin, glucagon, and other peptides. SRIF may affect key cellular processes in diverse tissues by regulating the release of growth factors and cytokines as well as cellular responses to these stimuli. SRIF can contribute to control of lymphocyte and inflammatory cell proliferation and activity, tumor cell growth, and normal tissue plasticity (Patel 1999; Rai et al. 2015). In the human skin, SRIF has been suggested as a negative regulator of epidermal wound healing (Vockel et al. 2011). Cortistatin binds to all SRIF receptors with similar affinity (Siehler et al. 2008) than SRIF and shares many functional properties with SRIF in the brain, within the hypothalamus-pituitary system and in the periphery (Spier and de Lecea 2000). Actually, cortistatin may activate GPCRs other than SSTs, i.e., the ghrelin receptor 1a (GHS-R1a) (Callaghan and Furness 2014).

Somatostatin Receptor Subtypes

Between 1990 and 1997, five distinct SRIF receptor subtypes were cloned in mice, rats, and humans. According to the International Union of Basic and Clinical Pharmacology (IUPHAR)

guidelines, these receptors are now referred to as SST_1 to SST_5 (Gunther et al. 2018) (Table 1). In the early 2000s, it was shown that some SSTs were capable of forming homodimers as well as heterodimers with other subtypes of the same family or with other GPCRs (Rocheville et al. 2000a, b; Pfeiffer et al. 2001, 2002). This kind of interaction between cell surface receptors may further enlarge the number of distinct receptor sites with differential functional properties for the same ligand. SSTs can be separated into two groups on the basis of their structural homologies and pharmacological properties. The first class comprises SST_2, SST_3, and SST_5. The second class comprises SST_1 and SST_4. All the SSTs are class A GPCRs that belong to the rhodopsin-like family of receptors (Table 1). They all possess seven transmembrane domains according to the structure of a prototypical GPCR. There is high sequence similarity between different subtypes and for a given subtype across different species. Moreover, all the five subtypes share some primary structural features:

- The conserved YANSCANPILY sequence in the seventh transmembrane domain is regarded as a mammalian SST signature.
- The DRY motif in the second intracellular loop is involved in coupling to G proteins.
- The consensus motif X-[S/T]-X-Φ at the end of the carboxyl-terminal tail is a potential PDZ domain binding site and is crucial for interaction with scaffolding proteins.
- N-glycosylation sites.

In recent years, variants of SST5 mRNA formed by splicing of non-canonical donor and acceptor splice sites have been identified in humans, pigs, and rodents. The human SST_5 variants may generate truncated receptors containing five or four transmembrane domains (SST_5TMD5 and SST_5TMD4, respectively) and distinct carboxyl termini (Durán-Prado et al. 2009), which display distinct tissue distribution and subcellular localization, compared with canonical full-length SST_5 (Córdoba-Chacón et al. 2011). The truncated receptors are highly expressed in tumor tissues and can interact with full-length SST_5 and SST_2 to retain them in intracellular vesicles. Hence, they may act as functional dominant-negative partners for the full-length receptors.

Canonical SSTs share many structural characteristics and also the main intracellular signaling pathways they are coupled to. All SSTs mediate the inhibitory actions of SRIF on endocrine and exocrine secretion and neuronal excitability by recruitment of G_i/G_o proteins, members of heterotrimeric, pertussis toxin (PTX)-sensitive guanine-nucleotide-binding proteins. The activation of G_i/G_o proteins by SSTs is coupled to the suppression of the second messenger cyclic AMP (cAMP) by inhibition of adenylyl cyclase activity and the decrease of cytosolic Ca^{2+} caused by inhibition of Ca^{2+} influx. To this end, SRIF can inhibit calcium channels both directly and indirectly by opening G-protein-activated-inward-rectifier K^+ channels (GIRK) to induce membrane hyperpolarization that leads to inhibition of Ca^{2+} influx via voltage-operated calcium channels (VOCC). The signaling events mediating the inhibitory effects of SRIF on cell proliferation are debated. The activation of protein tyrosine phosphatases (i.e., Src homology region 2 domain-containing phosphatase SHP-1 and SHP-2) may counteract growth factor-stimulated tyrosine kinase activity and inhibit multiple mitogenic pathways. The modulation of the extracellular-signal-regulated kinase (ERK) pathway may also play a role. Finally, the second class of SSTs and SST_3 is able to exert an inhibitory activity on sodium/hydrogen exchanger-1 (NHE1) via a PTX-insensitive mechanism. A decrease in NHE1 activity results in extracellular medium acidification that may be involved in inhibition of cell migration by SRIF (Buchan et al. 2002). SSTs can be better differentiated according to their expression in normal and tumor tissues, cellular localization, functional and pharmacological properties, and differential modes of activity regulation.

Agonist-Dependent Receptor Regulation

SRIF binding to its receptors results in desensitization, followed by internalization of receptor-

Somatostatin, Table 1 Class II and Class I somatostatin receptor subtypes: tissue distribution and role in physiology

Receptor	Expression in normal tissues	Expression in human tumors	Phenotype of mice lacking receptor	Receptor function
SST_1	Brain and spinal cord, anterior pituitary, pancreatic islets, GI tract Subcellular localization: when expressed in transfected HEK293 cells, localized to intracellular vesicles In primary cells and in sections from diverse human tumors, localized both at the plasma membrane and in the cytoplasm	GH adenomas, NET, prostate cancer	Altered insulin homeostasis	Presynaptic localization, autoreceptor in the CNS Within hypothalamus, inhibition of GHRH release and SRIF release Control of gastric emptying and colonic mobility Control of inflammatory response and nociception transmission Control of GH release from pituitary and insulin from beta-cells
SST_4	Brain, retina, dorsal root ganglia, placenta		Increased seizure susceptibility Increased anxiety Increased inflammatory and nociceptive response	Post-synaptic localization in the CNS Expressed in areas involved in learning and memory processes Involved in the modulation of behavioral and neuroendocrine response to acute and chronic stress (related to anxiety and depression-like behavior) Anticonvulsant effects Therapeutic target for development of anti-inflammatory and/or analgesic drugs without endocrine side effects (airway inflammatory diseases and bronchoconstriction)
SST_2	Brain (including cerebellum), spinal cord, retina, dorsal root ganglia, pituitary, GI tract, pancreatic islets, lymphatic tissue, adrenals (reticular zone), kidney	Brain tumors, GH adenomas, TSH adenomas, pheochromo-cytomas, NET	High basal acid secretion, inhibition of glucagon release, impaired motor coordination	Inhibitory neuromodulation in the CNS, main role in transduction of SRIF action in hippocampal formation Antiepileptic activity Antinociceptive activity Control of extrapyramidal motor system Increase of food intake and rapid-onset water consumption Control of stress response (autonomic and behavioral) Control of GH and TSH release from pituitary, glucagon, and insulin release from pancreatic islets Retinal neuroprotection

(continued)

Somatostatin, Table 1 (continued)

Receptor	Expression in normal tissues	Expression in human tumors	Phenotype of mice lacking receptor	Receptor function
SST_3	Brain, anterior pituitary, pancreatic islets, GI tract, lymphatic tissue, adrenal cortex and medulla, kidney Subcellular localization: primary cilia (but not restricted to) in neurons and endocrine cells	GH adenomas, ACTH adenomas, non-functioning adenomas	Impaired novel object recognition	Involved in object recognition memory mediated by primary neuronal cilia (rodent brain) Anticonvulsant activity Control of GH from pituitary, insulin release from pancreatic islets
SST_5	Anterior pituitary, pancreatic islets, GI tract, lymphatic tissue, adrenal cortex and medulla, kidney, parotid glands, thyroid (C cells) Subcellular localization: both at the plasma membrane and in the cytoplasm	GH adenomas ACTH adenomas Thyroid carcinomas, NET, breast, cervical, ovarian, and prostate carcinomas, differentiated colorectal cancers	Increased insulin secretion, basal hypoglycemia	Physiological control of pituitary function (GH, ACTH, TSH secretion) and endocrine pancreas activity (insulin secretion) SST5 displays constitutive activity (tonic inhibition of cAMP and ERK1/2 signaling) contributing to the control of pituitary hormone secretion

ligand complexes, a critical process that leads to receptor recycling to plasma membrane and resensitization or, alternatively, to receptor degradation in intracellular vesicle compartments. Regulation of GPCRs depends upon molecular events starting from phosphorylation at the cytoplasmic C-terminal tail by different GPCR kinases (GRKs), followed by recruitment of β-arrestins and receptor endocytosis. β-Arrestins may also act as scaffolding proteins involved in intracellular signaling cascades. Studying SST phosphorylation and trafficking has received great attention because the fate of internalized receptors following agonist exposure may affect cell responsiveness to endogenous ligands and drugs of therapeutic interest. The distinct SST subtypes undergo differential events upon agonist binding. Moreover, as to a given subtype, internalization and trafficking may differ across species (Tulipano and Schulz 2007).

SST_1 In heterologous cell system, rat SST_1 undergoes endocytosis and recycling upon SRIF binding, whereas human SST_1 displays very slow internalization despite rapid desensitization and uncoupling of adenylyl cyclase. One amino acid change at a putative phosphate-acceptor site (Thr383-Cys384-Thr385-Ser386) in the rat versus human receptor C-terminal tail (human Ser386 has been replaced by alanine in rat SST_1) may explain the differential interaction with β-arrestin1 and differential trafficking.

SST_2 This receptor subtype is almost completely confined to the plasma membrane. Agonist binding (SRIF, octreotide) causes multiple phosphorylation events within the C-terminal tail by GRK_2 and GRK_3 followed by receptor internalization. The following phosphorylation sites have been identified in both rat and human SST_2: S341, S343, T353, T354, T356, and T359. Unlike SRIF and octreotide, pasireotide (also known as SOM230) stimulates the phosphorylation of S341 and S343 residues only and causes partial receptor internalization (Lesche et al. 2009; Lehmann et al. 2014). The activation of SST_2 results in marked recruitment of both β-arrestin-1 and β-arrestin-2. The β-arrestin-dependent trafficking of the SST_2 somatostatin

receptor resembles that of a class B GPCR receptor (Oakley et al. 2000) in that upon receptor activation, β-arrestin and the receptor form stable complexes and internalize together into the same endocytic vesicles. Unlike other class B receptors, however, the SST_2 receptor undergoes rapid resensitization and recycling to the plasma membrane after activation by SRIF-14. Actually, receptor recycling is dependent on endosomal acidification and endothelin-converting enzyme-1 (ECE-1)-dependent degradation of the ligand. SRIF-28 and octreotide are resistant to cleavage by ECE-1. Hence, upon activation by these ligands, SST_2 remains in the intracellular vesicular compartment for a prolonged period of time (Zhao et al. 2013).

SST_3 A unique feature of this subtype is its long carboxyl-terminal tail. Upon agonist binding, the receptor is phosphorylated by GRK_2 and GRK_3. Phosphorylation sites have been identified in human and rat SST_3. The human SST_3 receptor is phosphorylated at S337, T341, T348, and S361 (Lehmann et al. 2016). The rat SST_3 receptor is phosphorylated at S341, S346, S351, and T357 (Roth et al. 1997). Receptor phosphorylation triggers β-arrestin recruitment and internalization. The β-arrestin mobilization of the SST_3 resembles that of a class A receptor in that upon receptor activation, β-arrestin and the receptor form relatively unstable complexes that dissociate at or near the plasma membrane. Unlike other class A receptors, SST_3 receptor undergoes substantial downregulation upon agonist exposure by ubiquitin-dependent lysosomal degradation and does not rapidly recycle to the plasma membrane (Tulipano et al. 2004; Lesche et al. 2009; Lehmann et al. 2016). Finally, synthetic analogs such as pasireotide and octreotide have been reported to act as partial agonists with regard to SST_3 phosphorylation and internalization compared to SRIF-14 (Lehmann et al. 2016).

SST_4 In heterologous cells, rat SST_4 is not subject to SRIF-induced phosphorylation or internalization and does not recruit β-arrestin. The absence of internalization was also shown in brain tissue after in vivo treatment with SRIF (Kreienkamp et al. 1998; Schreff et al. 2000). A single amino acid residue (Thr331) in the rat SST_4 sequence confers resistance to agonist-induced internalization (Kreienkamp et al. 1998). As to human SST_4, poor internalization occurs in transfected cells. A rapid dissociation of the SRIF-receptor complex and rapid recycling of the receptor to the plasma membrane has been suggested. Actually, in contrast to the rat receptor, human SST_4 signaling (NHE1 activation and ERK phosphorylation) was shown to be subject to sustained desensitization. Hence, this event can be regarded as not linked to receptor sequestration (Smalley et al. 2001).

SST_5 Two phosphorylation sites have been mapped in the SST_5 carboxyl-terminal tail. T347 is phosphorylated even in the absence of ligand, whereas T333 is phosphorylated by GRK2 in response to agonist binding and is involved in β-arrestin recruitment and receptor internalization. The third intracellular loop plays a key role in the interaction between the receptor and β-arrestin (Peverelli et al. 2008; Petrich et al. 2013; Schulz et al. 2014). Like SST_3, SST_5 forms relatively unstable complexes with β-arrestin, which rapidly dissociate before SST_5 trafficking to early endosomal vesicles. In contrast to SST_3 and SST_2, the proportion of SST_5 internalized after exposure to SRIF is rather low. The 36 terminal residues of the carboxyl-terminal tail are believed to contribute to the inhibition of receptor internalization. As to the internalized receptors, in heterologous cells, it has been shown that ligand-receptor dissociation leads to receptor recycling to the cell surface, accompanied by recruitment of receptors residing in an intracellular pool to the plasma membrane (Stroh et al. 2000). Finally, it should be noted that the phosphorylation and trafficking following to agonist binding to SST_5, are ligand- and cell context-dependent. SRIF-14 induces rapid phosphorylation, whereas octreotide does not. Pasireotide

has been shown to induce SST5 phosphorylation to a lesser extent than SRIF-14.

Drugs

Ligands

Receptor	Endogenous agonists	Synthetic agonists	Synthetic antagonists
SST_1	SRIF-14, SRIF-28; CST-17, CST-19	L-797,591 BIM-23926 Pasireotide	SRA880
SST_2	SRIF-14, SRIF-28; CST-17, CST-19	L-779,976 BIM-23120 Octreotide Lanreotide Pasireotide (partial agonist) Veldoreotide (somatoprim)	JR-11
SST_3	SRIF-14, SRIF-28; CST-17, CST-19	L-796,776 Octreotide Pasireotide	ACQ090 sst3-ODN-8 MK-4256
SST_4	SRIF-14, SRIF-28; CST-17, CST-19	NNC26-9100 L-803,087 J-2156 Veldoreotide (somatoprim)	
SST_5	SRIF-14, SRIF-28; CST-17, CST-19	L-817,818 BIM-23268 Octreotide Lanreotide Pasireotide (higher affinity vs other SSTs) Veldoreotide (somatoprim)	S5A1

SRIF Analogs in Clinical Practice

Octreotide and **lanreotide** are the two SRIF analogs still most widely used in clinical practice. Increased metabolic stability, potent suppressing activity on GH secretion, and low insulin suppressing activity have made them useful for treating acromegaly (Bauer et al. 1982; Heiman et al. 1987). SRIF analogs were also shown to reduce GH-secreting pituitary tumor size (Giustina et al. 2012). The partial dissociation of the inhibitory effects on GH and insulin secretion compared to native SRIF was explained with the identification of distinct SRIF receptor subtypes which can be differentiated in relation to their expression levels in normal and tumor tissues. Indeed, octreotide and lanreotide have greater affinity (subnanomolar) for SST_2 and moderate affinity for SST_5. Both receptors are involved in the control of GH secretion in humans. On the other hand, SST_5 plays a main role in the control of insulin secretion from ß-cells. If the rationale for developing SRIF analogs showing subtype selectivity to limit side effects is still valid, the concept of targeting multiple SSTs by a multisubtype-selective analog or targeting complexes formed by multiple receptors (heterodimers) is regarded as attractive for possible enhanced efficacy. Indeed, octreotide and lanreotide do not allow to achieve normalization of GH and IGF-1 levels in a significant percentage of acromegalic patients, and there is pre-clinical evidence that greater SST_5 activation may lead to a better control of GH hypersecretion in patients which are only partially responsive to these analogs. BIM-23244 can be regarded as a bi-selective analog targeting SST_2 and SST_5. Actually, its high activity at SST_5 discouraged its development for the risk of unwanted pancreatic side effects. Veldoreotide is a ligand with moderate affinity for multiple subtypes (SST_2, SST_5, and SST_4) and is in phase 2 trials for the treatment of acromegaly. Finally, the concept of targeting the interaction between receptors has been extended to multi-ligands able to interact with a given SST subtype and receptors outside of the SST family, i.e., dopamine receptor subtype-2 (D_2). So-called chimeric dopastatins are no longer in development.

Pasireotide is a cyclohexapeptide with high affinity for SST_1, SST_2, and SST_3 and subnanomolar affinity for SST_5. Pasireotide is called a pan-receptor-specific ligand. Pre-clinical and clinical data suggest that pasireotide may be helpful to achieve control of GH secretion in a subgroup (15–20%) of patients resistant to octreotide or lanreotide. In detail, pasireotide can be more effective than octreotide in tumors showing lower expression of SST_2 and lower SST_2/SST_5 ratio

(Gadelha et al. 2014; Gatto et al. 2017). Moreover, in contrast to lanreotide and octreotide, pasireotide has shown potential efficacy in a subset of patients with Cushing's disease. However, in keeping with its high affinity at SST_5, pasireotide may worsen glucose control related to insulin resistance in both acromegaly patients and in Cushing's disease, and monitoring blood glucose and electrocardiogram is important.

Disease	Analog	Therapeutic effects
Acromegaly	Lanreotide Octreotide Pasireotide (long-acting formulations for i.m. or s.c. administration)	Biochemical control (decreased GH and IGF-1 levels) Decreased tumor size Beneficial impact on disease comorbidities (determined by age, degree of achieved biochemical control, duration of the disease)
Cushing's disease	Pasireotide	Decreased urinary-free cortisol (UFC) Decreased mean tumor size Beneficial impact on symptoms related to hypercorticolism (however, blood glucose and glycated hemoglobin levels may increase)
Neuroendocrine tumors (carcinoids, GI and pancreatic NETs)	Lanreotide Octreotide (long-acting formulations)	Symptomatic improvement due to suppression of hormone release Decreased or stabilized tumor mass Improved survival

The efficacy rates of therapies with SRIF analogs vary depending on tumor type and individual patient. As to GH-secreting pituitary tumors, some tumors are resistant to SRIF analogs. Moreover, SRIF analogs do not enable GH and IGF-1 normalization in a subset of responsive patients. To this end, a personalized approach to acromegaly classification and management has been proposed. Adverse determinants of SRIF analog responsiveness include advanced age, tumor size, levels of GH and IGF-1, and a series of molecular markers in pituitary tumor cells (Cuevas-Ramos et al. 2015). Corticotroph tumors are usually resistant to octreotide and lanreotide. About 20% of patients with Cushing's disease achieve biochemical normalization with pasireotide (Colao et al. 2012). Tachyphylaxis has not been observed in acromegaly, but it has been described at variable time intervals in patients harboring NETs.

SRIF-Based Radiopharmaceuticals

Radiolabelled somatostatin analogs are used for tumor imaging (peptide receptor-targeted scintigraphy) and peptide receptor radionuclide therapy (PRRT) of neuroendocrine tumors. A radioiodinated analog of octreotide ($[^{123}I]Tyr^3$-octreotide) was synthetized in 1989 (Krenning et al. 1989) and allowed the first successful non-invasive imaging of SST-rich tumors. This compound can be regarded as a starting point for the development of radiolabeled ligands for SST-targeted imaging and radionuclide therapy. Current ligands include a chelator conjugated to the N-terminus of a peptide structure derived from SST-targeting drugs (i.e., octreotide or lanreotide). The chelator (DTPA, DOTA, NOGADA) ensures the stable complexation of radiometals (^{68}Ga, ^{111}In, ^{177}Lu, ^{90}Y). These compounds exhibited agonistic behavior and high affinity for SST_2 (^{111}In-DTPA-OC, ^{68}Ga-DOTA-TOC, ^{68}Ga-DOTA-TATE, ^{177}Lu-DOTA-TATE). Radiolabelled ligands that bind with high affinity to multiple SST receptor subtypes are in development (^{68}Ga-DOTA-NOC). Metabolic stability, low lipophilicity, renal clearance, and absence of accumulation in the upper abdominal region due to hepatobiliary excretion are favorable properties of currently applied radiolabeled SRIF analogs compared to radioiodinated octreotide. The high sensitivity of SST-targeted has been assumed to be dependent on internalization of the receptor after

radioligand binding that leads to retention of radiometals in target cells. Nevertheless, in the last decade, evidence has accumulated that SST-targeted imaging with radiolabelled antagonists, which poorly internalize, can be more sensitive and effective than that employing corresponding receptor agonists that are highly internalized. The molecular mechanism underlying tumor imaging performance using SST antagonists needs to be elucidated. Actually, it has been suggested that a full antagonist can label receptors in all states (active or inactive), whereas a corresponding agonist binds to an active conformation, only.

Cross-References

► Peptides and Peptidomimetics as Foundations for Drug Discovery

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Specialized Pro-resolving Lipid Mediators

► Pro-resolving Mediators

Sphingosine-1-Phosphate

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Definition

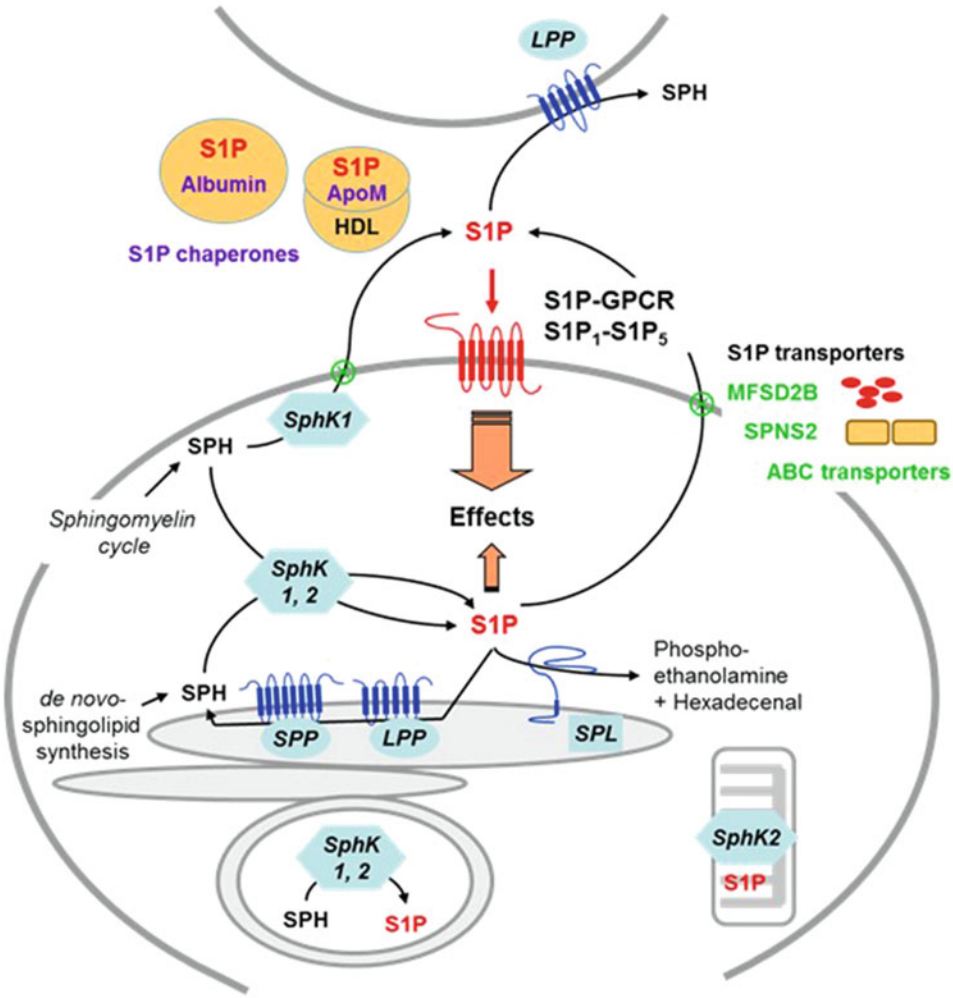
Sphingosine-1-phosphate is a lysophospholipid, and product of sphingolipid metabolism.

Basic Characteristics

Metabolism and Occurrence

Sphingosine-1-phosphate (S1P) is formed by phosphorylation of sphingosine by sphingosine kinases (SphK1,2; human gene names, *SPHK1,2*) (Chan and Pitson 2013; Pyne et al. 2016). Prototypical S1P d18:1 contains the (E,2S,3R)-2-aminooctadec-4-ene-1,3-diol sphingoid base backbone (D-erythro-C18-sphingosine). This structure results from sequential reactions starting with condensation of serine and palmitoyl-CoA by serine palmitoyltransferase (SPT; human gene names, *SPTLC1-3*). The double bond is introduced by delta-4-desaturases (human gene names, *DEGS1,2*) (Maceyka and Spiegel 2014; Obinata and Hla 2019). Under certain conditions, SPT may also use other amino or fatty acids and thereby produce sphingoid base backbones with other chain lengths and substitutions. Degradation of S1P occurs reversibly by non-selective lipid phosphate phosphatases, $LPP_{1–3}$, or by selective S1P phosphatases ($SPP_{1,2}$). S1P lyase (human gene name, *SGPL1*) cleaves S1P irreversibly (see Lysophospholipids, Fig. 1b).

S1P is usually generated intracellularly and needs to be exported for interaction with its specific G-protein-coupled receptors (S1P-GPCR; Fig. 1). SphK1 and SphK2 differ in tissue expression and subcellular localization, regulation and function. Their expression and activity are regulated in diverse ways, for example, by growth factors, cytokines, or GPCR agonists (Chan and Pitson 2013; Pyne et al. 2016). Some stimuli induce a translocation of SphK1 from the cytosol to the plasma membrane, enabling S1P secretion and so-called inside-out signalling. Both SphK isoforms can occur in cytosol and nucleus; however, SphK2 is more abundant in the nucleus and also found at the ER and in mitochondria. Nuclear and mitochondrial SphK2 address intracellular effectors of S1P. These may be, for example, histone deacetylases or prohibitin-2 (Pyne et al. 2016; Spiegel et al. 2019), but these targets still await independent confirmation. S1P excretion is mediated by sphingolipid transporter-2 (SPNS2), major facilitator superfamily domain containing 2B (MFSD2B) and several ATP-binding cassette



Sphingosine-1-Phosphate, Fig. 1 The S1P signalling system. *ApoM* apolipoprotein M, *HDL* high-density lipoprotein, *LPP* lipid phosphate phosphatase, *MFSD2B* major facilitator superfamily domain containing 2B, *SPH* sphingosine, *SphK* sphingosine kinase, *SPL* S1P lyase, *SPNS2* spinster homologue-2, sphingolipid transporter-2, *SPP* S1P phosphatase

(ABC) transporters (Spiegel et al. 2019; Riboni et al. 2020). S1P occurs in plasma at high concentrations (~0.2–0.4 μM). Main sources of circulating S1P are erythrocytes expressing MFSD2B and endothelial cells expressing SPNS2 (Obinata and Hla 2019). S1P is furthermore stored in platelets, released via MFSD2B upon platelet activation, and occurs in serum at ~0.5–0.8 μM. About 65% of plasma S1P is bound to apolipoprotein M (ApoM) within high-density lipoproteins (HDL); the remainder is bound to albumin. These S1P chaperones fine-tune S1P's interaction with S1P-GPCR, with ApoM producing a sustained S1P release (Obinata and Hla 2019). Of note, tissue S1P levels are very low, and there are S1P gradients between tissues and circulation and within tissues. These chemotactic gradients are essential for trafficking of immune cells and positioning of cells within tissues (Dixit et al. 2019).

S1P Receptors

An overview of G protein-coupled S1P receptors as listed in the IUPHAR/BPS Guide to Pharmacology is presented in Table 1. These receptors

Sphingosine-1-Phosphate, Table 1 G protein-coupled S1P receptors

Receptor (previous names)	Signalling	Main biological effects
$S1P_1$ (EDG1)	$G_{i/o}$	Cell migration ↑, cell proliferation, survival, cell-cell contacts, lymphocyte trafficking, angiogenesis, vascular barrier ↑, heart rate and atrioventricular conduction ↓ in humans
		-/- mice: embryonic lethality, defective vascular maturation
$S1P_2$ (EDG5, H218, AGR16)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$	Cell migration ↓, vascular barrier ↓, vascular development
		-/- mice: hearing loss, B cell lymphoma at high age, neuronal hyperexcitability
		Human mutations: hearing impairment
$S1P_3$ (EDG3)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$	Vascular development, NO-dependent vasorelaxation, heart rate ↓ in mice
		-/- mice: no obvious abnormalities
$S1P_4$ (EDG6)	$G_{i/o}$, $G_{12/13}$	Expression limited to haematopoietic tissues. Anti-inflammatory activity, proliferation and cytokine secretion of T-cells ↓, 5-lipoxygenase ↓, terminal megakaryocyte differentiation
		-/- mice: delayed recovery from thrombocytopenia
$S1P_5$ (EDG8, NGR-1)	$G_{i/o}$, $G_{12/13}$	Cell proliferation, mitotic progression, cell rounding, oligodendrocyte functions, natural killer cell trafficking
		-/- mice: altered natural killer cell homing and tissue invasion

comprise the class A GPCR and $S1P_{1–5}$ (human gene names, *S1PR1-5*) (see the IUPHAR GPCR database). S1P-GPCR differentially couples to $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ proteins (Table 1). Thus, these receptors regulate classical signalling pathways such as ▶ adenylyl cyclase, phospholipase C $[Ca^{2+}]_i$, protein kinases C, mitogen-activated protein kinases, Akt, Ras, Rac and Rho. Thereby, they modulate cell growth, survival, re-arrangement of the cytoskeleton, cell migration, Ca^{2+} signalling and other responses. The $G_{12/13}$-coupled receptors also activate the Hippo pathway, which controls cell growth, immunity, fibrosis and cancer (Noguchi et al. 2018). $S1P_{1-3}$ are widely expressed, while $S1P_4$ is rather restricted to haematopoietic tissues and $S1P_5$ is preferentially expressed in the brain, immune cells, skin and other tissues. Functional studies have demonstrated that also S1P can act independently of the known S1P-GPCR. Several intracellular targets for S1P have been described but still await independent confirmation (Pyne et al. 2016; Spiegel et al. 2019).

Biological Actions

The components of the S1P signalling system are widely expressed. They regulate tissue homoeostasis in many organs, including the heart, kidney, liver, intestine, pancreas, adipose tissue, bone, skin and immune and nervous systems.

Development The S1P signalling system plays a role in angiogenesis, neurogenesis, and limb development in mice and regulates cardiogenesis in zebrafish (Mendelson et al. 2014). Mice lacking *S1pr1* die during embryonic development from haemorrhages caused by a failure of pericytes to migrate around newly formed capillaries. Additional deletion of *S1pr2* or *S1pr3* leads to more severe vascular defects. Mice lacking both SphK isoforms do not have measurable S1P concentrations and die during embryonic development from haemorrhages and defective vasculogenesis. Furthermore, they exhibit disturbed neurogenesis and impaired neural tube closure. Defective neurogenesis is also observed in *S1pr1* knockouts, indicating that the *Sphk/S1pr1* axis is important for angio- and neurogenesis (Mendelson et al. 2014). Both the $S1P_2$ receptor and SPNS2 are essential for function of the inner ear. Mice lacking *S1pr2* or *Spns2* suffer from hearing loss early after birth, and mutations of *S1PR2* in humans are associated with deafness (Romero-Guevara et al. 2015). Furthermore, both the $S1P_2$ receptor and SPNS2 are required for migration of cardiac precursors to the midline in zebrafish (Mendelson et al. 2014). Finally, deletion of *Sgpl1*, which represents the only exit point of sphingolipid metabolism, leads to multiple organ defects and early death in mice. Similarly, loss-of-function mutations in *SGPL1*

cause S1P lyase insufficiency syndrome (SPLIS) in humans, with steroid-resistant nephrotic syndrome, damage of the central and/or peripheral nervous systems, adrenal insufficiency and other symptoms (Choi and Saba 2019).

S1P Gradients and the Immune System SphK, LPP, S1P lyase and SPNS2 contribute to formation of S1P concentration gradients between tissues and circulation and within organs (Dixit et al. 2019). S1P-GPCRs regulate cell migration and positioning within these gradients, with $S1P_1$ and $S1P_2$ acting antagonistically. $S1P_1$ activates Rac and stimulates migration, while $S1P_2$ activates Rho, induces stress fibre formation and inhibits migration. This is of particular importance for immune cell trafficking: $S1P_1$ is required for lymphocyte egress from lymphatic tissues, while $S1P_2$ mediates confinement of B cells and follicular T helper cells to lymph node germinal centres. The $S1P_5$ receptor is essential for natural killer cell trafficking. While S1P regulates migration of many types of immune cells, the S1P signalling system also regulates development, differentiation and polarization of these cells (Blaho and Hla 2014; Weigert et al. 2019).

Cardiovascular System S1P is an important mediator in the cardiovascular system. Not only angio- and vasculogenesis but also vascular permeability is regulated by S1P: $S1P_1$ stimulates endothelial cell adherens junction assembly and improves the vascular barrier, while $S1P_2$ disrupts adherens junctions and induces vascular leak (Obinata and Hla 2019). The $S1P_2$ receptor induces contraction of diverse types of smooth muscle, including vascular, bronchial, intestinal and bladder smooth muscle, via $[Ca^{2+}]_i$ increases and activation of Rho/Rho kinase (Blankenbach et al. 2016). Endothelial $S1P_1$ and $S1P_3$ receptors, on the other hand, cause NO-dependent vasorelaxation. S1P has both pro- and anti-atherosclerotic effects (Kurano and Yatomi 2018). $S1P_2$ retains macrophages in atherosclerotic plaques, and knockout of this receptor was beneficial in models of atherosclerosis. $S1P_1$ acts anti-atherosclerotic via activation of NO synthase and suppression of endothelial adhesion molecules and is preferentially activated by ApoM-bound S1P (Kurano and Yatomi 2018). $S1P_3$ in mice and $S1P_1$ in humans, respectively, activate $I_{K(ACh)}$ in atrial myocytes, leading to bradycardia and delay of atrioventricular conduction (Dyckman 2017).

Inflammation, Fibrosis and Cancer The S1P signalling system also affects **inflammation, fibrosis and cancer**. This is due to S1P's role in cell proliferation, cell survival, migration and invasion and its eminent effects on cells of the innate and adaptive immune systems. In particular, the importance of $S1P_1$ for lymphocyte trafficking makes it a target in many inflammatory and autoimmune diseases, such as multiple sclerosis, inflammatory bowel disease, psoriasis, lupus erythematosus and others (see Drugs). Key pro-inflammatory mediators, for example, interleukin-1β and tumour necrosis factor-α, activate and induce SphK (Chan and Pitson 2013), which can lead to either intracellular S1P formation or S1P export and cross-activation of S1P-GPCR. Extracellular S1P can act both pro- and anti-inflammatory (Maceyka and Spiegel 2014; Nagahashi et al. 2018). For example, the $S1P_2$ receptor activates NF-κB and/or induces cyclooxygenase-2 and prostaglandin production in several cell types (Blankenbach et al. 2016). Also the key mediator of fibrosis, transforming growth factor-β (TGF-β), induces SphK1. In inflammatory kidney diseases, SphK1 is often upregulated and appears to act as a brake in kidney fibrosis (Huwiler and Pfeilschifter 2018). Extracellular S1P, on the other hand, can cross-activate the TGF-β/Smad signalling cascade and increase the production of extracellular matrix (Huwiler and Pfeilschifter 2018). In cancer, S1P affects both tumour cells and cells of the tumour microenvironment (Pyne et al. 2018; Riboni et al. 2020).

Drugs

There are currently three drugs approved that target the S1P signalling system: fingolimod and ozanimod for relapsing-remitting multiple sclerosis and siponimod for secondary progressive multiple sclerosis. Fingolimod (FTY720), which structurally resembles sphingosine, is phosphorylated by SphK2

Sphingosine-1-Phosphate, Table 2 Drugs targeting the S1P signalling system

Drug	Target	Indication	Stage
Fingolimod	S1P receptor modulator	Relapsing-remitting multiple sclerosis	Approved
	$S1P_{1/3/4/5}$		
Siponimod (BAF-312)	S1P receptor modulator	Secondary progressive multiple sclerosis	Approved
	$S1P_{1/5}$		
Ozanimod (RPC1063)	S1P receptor modulator	Relapsing-remitting multiple sclerosis	Approved
	$S1P_{1/5}$	Ulcerative colitis	Phase III
Etrasimod (APD334)	S1P receptor modulator	Ulcerative colitis	Phase III
	$S1P_1$ ($S1P_4$, $S1P_5$)	Atopic dermatitis	Phase II
Ponesimod (ACT-128800)	S1P receptor modulator	Multiple sclerosis	Phase II
	$S1P_1$ ($S1P_3$, $S1P_5$)	Chronic plaque psoriasis	Phase II
Cenerimod (ACT-334441)	S1P receptor modulator	Systemic lupus erythematosus	Phase II
	$S1P_1$ (selectivity unknown)		
Amiselimod (MT-1303)	S1P receptor modulator	Relapsing-remitting multiple sclerosis, Crohn's disease, psoriasis, systemic lupus erythematosus	Phase II
	$S1P_1$ ($S1P_5$)		
Ceralifimod (ONO-4641)	S1P receptor modulator	Relapsing-remitting multiple sclerosis	Phase II
	$S1P_{1/5}$		
Mocravimod (KRP-203)	S1P receptor modulator	Ulcerative colitis, cutaneous lupus erythematosus	Phase II
	$S1P_{1/5}$		
Opaganib (ABC294640)	SphK2 inhibitor	Pancreatic cancer, unspecified adult solid tumour	Phase I

and then acts as agonist at all S1P-GPCR except $S1P_2$. The immunosuppressive activity of fingolimod is caused by super-agonistic activation and subsequent downregulation of the $S1P_1$ receptor which is required for lymphocyte egress from lymphatic tissues (Huwiler and Zangemeister-Wittke 2018). Lymphopenia induced by loss of $S1P_1$ represents fingolimod's principle mechanism of action in multiple sclerosis. Undesired effects include bradycardia and atrioventricular block induced by $S1P_1$-mediated activation of cardiac G protein-coupled inwardly rectifying potassium channels, macular oedema caused by disturbance of the vascular barrier, mild increase in arterial blood pressure, bronchoconstriction, increases in liver enzymes and elevated risk for infections (Huwiler and Zangemeister-Wittke 2018). The risk for bradycardia is high during the initial phase of therapy and declines with $S1P_1$ desensitization. Siponimod has a high selectivity for $S1P_1$ and $S1P_5$ and a shorter half-life compared to fingolimod. Despite sparing $S1P_3$, it still causes adverse effects in the cardiovascular system (Al-Salama 2019). Several other S1P receptor modulators with greater S1P-GPCR subtype selectivity are in clinical development (Dyckman 2017) (Stepanovska and Huwiler 2020); some of them are shown in Table 2. All act as super-agonists/functional antagonists at the $S1P_1$ receptor and thereby induce immunosuppression. Consequently, these compounds are developed for diverse autoimmune diseases (Table 2). While pharmaceutical development thus strongly focuses on $S1P_1$, other S1P-GPCR and SphK1/2 might as well be worthwhile targets. However, the only SphK inhibitor in clinical development is ABC294640, a SphK2 inhibitor with its K_i in the micromolar range, which is being evaluated as anticancer agent (Table 2).

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SPMs

▶ Pro-resolving Mediators

Statins

▶ Lipid-Lowering Drugs

Steroids

▶ Glucocorticoids

Stimulant Drugs

▶ Psychostimulants

Stimulants

▶ Psychostimulants

Stress Proteins

▶ Chaperones

Stroke

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Definition

Traditionally, stroke has been defined according to its clinical presentation as a focal neurological deficit with a sudden onset that probably has a vascular cause. The advent of computer tomography, magnetic resonance imaging (MRI), and other imaging techniques has allowed a stronger focus on the cerebrovascular pathology and has led to a broadening of the definition. Now, it includes ischemic infarcts and intracerebral hemorrhages, both with and without symptoms, transient ischemic attacks, subarachnoid hemorrhages, and cerebral venous thrombosis (Sacco et al. 2013). Global cerebral ischemia is usually excluded from the definition of stroke as it differs in clinical presentation and is caused by conditions, such as cardiac arrest or hypotensive shock, that lie outside of the cerebrovascular system.

Its high incidence and mortality make stroke one of the most important causes of death worldwide. The most common form of stroke is an ischemic infarction that is about four times as frequent as intraparenchymal hemorrhage. Based on the vascular origin, ischemic strokes are classified as cardioembolic, atherosclerotic, microvascular, and cryptogenic, each of which represents approximately a quarter of cases (Saver 2016). Additional classification builds on the involved vascular territory or the area of the CNS, including retina and spinal cord. Hypertensive intracerebral hemorrhages occur at preferred sites, such as the basal ganglia and the pons. Outside of these predilection sites, hemorrhages often have another aetiology. In the case of subarachnoid hemorrhage and cerebral venous thrombosis, the identification of an aneurysm or the site of thrombosis is important for the exact definition of the disease.

Basic Mechanisms

Ischemic Penumbra

All forms of stroke are associated with focal ischemia in the brain, spinal cord, or retina. Even in hemorrhagic strokes, compression of vessels or vasospasms leads to secondary ischemia of the tissue contributing to the neurological deficits. However, in contrast to global ischemia, focal ischemia is associated with some residual blood flow through collateral vessels, which provides a route for drugs to reach the lesion. Low perfusion in the core of the ischemia triggers rapid tissue loss. In the border zone between core and healthy tissue, blood flow gradually increases. The tissue, in which blood flow is high enough for acute survival but still so low to put it at risk for subacute demise, is called penumbra. Salvaging the penumbra is the target of stroke therapy. Size and distribution of the penumbra are dynamic and depend on individual factors, such as collateral blood vessels. For patient care, it is therefore important that MRI techniques are available that allow determining a proxy of the penumbra.

Pathomechanisms of Ischemic Brain Damage

Ischemia has a profound impact on the CNS. Four complexes in the pathophysiology explain the high vulnerability to ischemia: periinfarct depolarization, excitotoxicity, regulated cell death, and inflammation.

The CNS spends a lot of energy to maintain the polarization of neurons. Due to the loss of cellular energy in cerebral ischemia, neurons depolarize. A wave of **depolarization** spreads into the penumbra (Dreier 2011). There, the attempt of cells to repolarize strains the energy balance of the tissue that is already at risk due to the reduced perfusion. Depolarization of cells is associated with a shift of extracellular water into cells. This phenomenon is the basis of diffusion-weighted MR imaging that is used in clinical practice to detect early signs of cerebral ischemia. Lesions identified in diffusion-weighted MRI represent the core infarct area in which tissue usually does not recover.

Due to the energy loss in the ischemic tissue, excitatory neurotransmitters, mainly glutamate, are released and their re-uptake is impaired. This

is the basis of **excitotoxicity**. Activation of glutamate receptors, particularly those outside of synapses, triggers a detrimental cascade of events. Accordingly, the inhibition of glutamate receptors is neuroprotective in experimental stroke models, but clinical trials did not show an effect (Lai et al. 2014). A key problem in translating this and other findings seems to be the therapeutic time window in preclinical studies that is often too short for clinical practice. Targeting the events downstream of glutamate receptor activation might help to extend the therapeutic window. The stimulation of glutamate receptors leads to a calcium overload of cells and mitochondria. This damages mitochondria and enhances the production of reactive oxygen species. Another important consequence of glutamate receptor activation is the synthesis of NO by the neuronal NO synthase. This enzyme is coupled by the adaptor protein PSD-95 to glutamate receptors and is activated by elevated intracellular calcium levels. NO and superoxide anions form the highly reactive peroxynitrite that damages numerous macromolecules.

The death of neural cells denotes an irreversible step in the ischemic cascade. Evidence that many cells succumb to some form of **regulated cell death** raised hope that an intervention is possible. Apoptosis, necroptosis, autophagy, ferroptosis, and pyroptosis have all been reported in stroke models. Variants of cell death are often interrelated and blocking one form promotes another. For example, caspase inhibitors reduce apoptosis and ameliorate ischemic damage, but the effect seems to be dampened by enhanced necroptosis. Also, necroptosis inhibitors are protective in cerebral ischemia suggesting that a cocktail of cell death inhibitors may have a superior effect.

Dying cells release damage-associated molecular patterns, such as HMGB1 or ATP, that elicit a sterile **inflammation** in the CNS. Damage-associated molecular patterns act on microglia and other neural cells. In all cells of the CNS including neurons, cerebral ischemia stimulates pro-inflammatory signalling, such as the NF-κB pathway. In addition, neutrophils, monocytes, and lymphocytes are allured to the ischemic tissue. The complex tissue response has both detrimental and beneficial effects. On the damage side, inflammatory cytokines contribute to the demise of neurons. They also open the blood-brain barrier and promote vascular brain edema. In its worst form, brain edema critically elevates the intracranial pressure lowering cerebral perfusion and causing herniation and death of patients. On the protection side, activated microglia or brain macrophages reduce the damage by counteracting excitotoxicity, releasing anti-inflammatory mediators and removing tissue debris. The line between damage and protection seems to be fluid. Local properties including perfusion or systemic factors, such as body temperature, have a profound influence. In addition, timing is very important after stroke. Anti-inflammatory interventions may protect in the acute phase but impair recovery when administered too late (Lo 2008).

Pharmacological Intervention/ Pharmacotherapy

When treating patients with ischemic stroke, the first aim is to **reinstall perfusion**. Since 1996, tissue plasminogen (tPA, alteplase) has been used for thrombolysis and has recently been supplemented by mechanical intravascular thrombectomy. Often intravenous thrombolysis and intravascular thrombectomy are combined in individual patients. tPA variants with enhanced fibrin selectivity and prolonged half-life, such as tenecteplase, may be superior, but in 2020, alteplase is still the only approved agent for thrombolysis within 4.5 h after symptom onset. Imaging of the ischemic territory allows extending the therapeutic window in selected cases (Thomalla et al. 2018). However, with increasing time since onset of ischemia, the efficacy of thrombolysis decreases while the risk for intracerebral hemorrhage rises. Despite successful recanalization of occluded arteries, cerebral perfusion may remain low. This phenomenon, called no reflow, is attributed to a disturbance of the microvasculature. Apparently, small vessels are obstructed by constricting pericytes, swollen

astrocytes, or clogging immune cells (Bai and Lyden 2015). A disturbance of the microvasculature is also a likely cause of intracerebral hemorrhage that can be life-threatening after thrombolysis. Depending on local settings, up to 20% of patients with acute ischemic stroke are eligible for thrombolysis.

Besides acute recanalization therapy, **secondary prevention** is the most successful treatment option for stroke patients. After a stroke or transient ischemic attack, the risk for another event is elevated for several weeks. If cardiac embolism is not the cause of stroke, inhibitors of platelet aggregation significantly lower the risk of recurrence. The standard therapy is aspirin. Clopidogrel or aspirin plus dipyridamole are slightly more effective. Aspirin is also transiently combined with clopidogrel to further enhance its efficacy. Whether other P_2Y_{12} antagonists, like prasugrel or ticagrelor, could provide advantages is the subject of ongoing trials. In patients with cardioembolic stroke, anticoagulant drugs are administered to prevent a secondary event. In the very acute phase of ischemic strokes, anticoagulants are associated with an increased risk for intracerebral hemorrhages. Therefore, a lag time of 4–14 days between stroke onset and start of the anticoagulant therapy is recommended. In the treatment of patients with intracerebral hemorrhage, lowering high blood pressure is beneficial to reduce further bleeding (Moullaali et al. 2019). This is also the rationale why the blood pressure should be below 185 mmHg systolic and 110 mmHg diastolic before recanalization therapy in ischemic stroke. For the acute control of hypertension, the competitive α1 and β adrenergic receptor antagonist labetalol and the dihydropyridine calcium channel blocker nicardipine are used. After the acute phase, a strict control of hypertension has proven to be most effective in lowering the stroke rate. Finally, lipid-lowering drugs, mainly potent HMG-CoA reductase inhibitors, have an established place in the secondary prevention of ischemic stroke. Statins are afflicted with a low risk of causing intracerebral hemorrhages that is, however, outweighed by their beneficial effects in the presence of atherosclerotic diseases (Goldstein et al. 2008).

Future Directions

Stroke has served as a model disease for neurodegenerative disorders. Its pathophysiology has been intensively studied leading to an impressive armamentarium of neuroprotective drugs that work in preclinical experiments. However, translation of neuroprotection into clinical treatments proved to be difficult. In contrast, thrombolysis was more successful and shows that pharmacotherapy of ischemic stroke is possible. Therefore, an obvious aim is to improve thrombolysis further. Optimizing the pharmacokinetics or the fibrin selectivity of thrombolytics as well as limiting its inhibition by the plasma proteins PAI-1 and TAFI may increase the recanalization rate in acute stroke. In addition to plasminogen, alteplase cleaves NMDA receptors and basement membrane proteins which may contribute to its adverse effects. Limiting off-target cleavage may improve its safety profile (Thiebaut et al. 2018).

Blood coagulation is closely linked to inflammation and cell death. Along these lines, it may be possible to extend the success of anticoagulants and thrombolytics and to develop agents that manipulate cell death and inflammation in addition to blood coagulation. Activated protein C and thrombomodulin are endogenous factors that have this potential dual activity. Many inflammatory factors proved to be Janus-faced with both beneficial and detrimental actions. However, this is unlikely to be a universal principle and further anti-inflammatory strategies await to be tested. Other appealing targets are proteins that induce both apoptosis and necroptosis, a possible example being RIPK1.

To evaluate the potential of these and other targets, preclinical models should investigate the **long-term functional outcome** that is essential for clinical efficacy. Clinical trials usually evaluate the neurological deficit over 90 days. In contrast, this aspect has often been neglected in basic research. Long-term recovery is determined by post-insult plasticity and regeneration. The pharmacology of these processes is still largely unexplored but may be worthwhile to study.

In the future, progress may come from a **precision medicine** approach towards stroke. So far, clinical studies have often lumped all stroke patients into heterogeneous groups, irrespective of the aetiology, the size of the penumbra, or the presence of inflammation. New imaging techniques or biomarkers may help identify stroke patients that benefit most from specific therapies. NMR- and mass spectrometry-based lipidomics and metabolomics provide unprecedented insight into the molecular events underlying injury in individual patients and may increase therapeutic precision in the future.

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Structural Bioinformatics

▶ Molecular Modelling

Strychnine-Sensitive Glycine Receptor

▶ Glycine Receptors

sUA

▶ Physiology and Treatment of Hyperuricemia and Gout

Substance P

▶ Peptides and Peptidomimetics as Foundations for Drug Discovery

Substance P Receptor

▶ Neurokinin/Tachykinin Receptors

Substantia Nigra

▶ Dopamine System

Synapse

▶ GABAergic System

Synaptic Transmission

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Synonyms

Neurotransmission

Definition

Synaptic transmission is the transfer of biological information between two neurons (Fig. 1) (Chua et al. 2010). Major events during synaptic transmission are as follows:

(1) An action potential arrives at the presynaptic axon terminal.
(2) The ensuing depolarization triggers the opening of voltage-dependent Ca^{2+} channels (VGCCs).
(3) The increase of Ca^{2+} concentration in the axon terminals triggers the exocytosis of synaptic vesicles.
(4) The neurotransmitter released from the vesicles diffuses through the synaptic cleft.
(5) The neurotransmitter activates receptors in the plasma membrane of the postsynaptic neuron.
(6) Activated postsynaptic receptors mediate ion fluxes through the plasma membrane of the postsynaptic neuron, leading to changes in the membrane potential. The postsynaptic receptors may also mediate manifold biochemical changes within the postsynaptic neuron.

There are numerous transmitter substances. They include the amino acids glutamate, GABA, and glycine; acetylcholine; the monoamines dopamine, noradrenaline, and serotonin; the neuropeptides; and ATP. Nitrergic transmission, i.e., transmission by NO, differs from transmission by other transmitters and is not covered in this essay. Chemical synaptic transmission is by far the dominating kind of synaptic transmission in the human body. However, in a few regions of the nervous system, electrical synapses, based on gap junctions, can mediate bidirectional communication between adjacent neurons (Alcami and Pereda 2019).

Drugs that influence synaptic transmission play an eminent role in therapy, for two reasons. First, the nervous system controls all tissues. Second, with few exceptions synaptic transmission is chemical, operating by means of transmitter substances, and synapses therefore provide a large number of drug targets, for example, transporters and enzymes that remove transmitters from the synaptic cleft. Poisons like sarin and cocaine also target synaptic transmission.

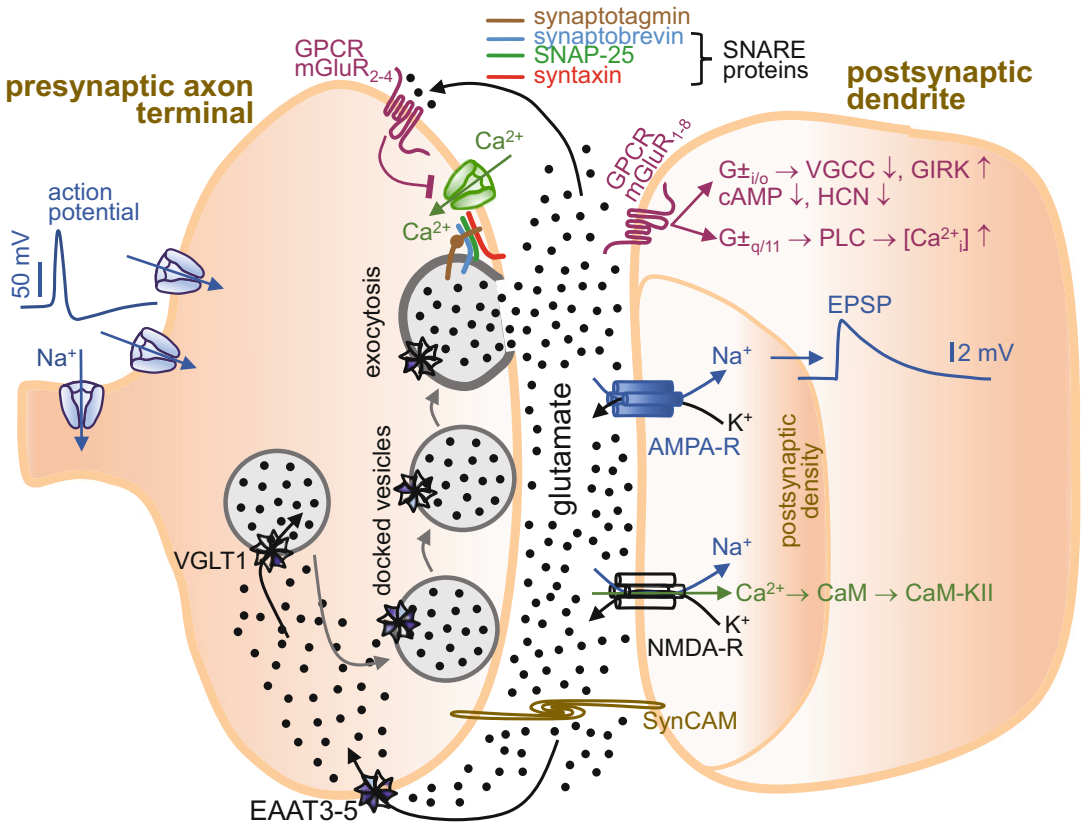
Basic Mechanisms

Transmitter Synthesis and Vesicular Storage

All non-peptide neurotransmitters are produced in the axon terminals, which possess the necessary enzymatic machinery. After synthesis, most of the transmitters are brought into synaptic vesicles by transporters belonging to the solute carrier transporter family (SLC transporters) (Fig. 1). The vesicular SLC transporters mostly utilize the proton electrochemical gradient across the vesicle membrane.

Glutamate and GABA

In neurons glutamine is transformed into glutamate by the enzyme glutaminase. After synthesis glutamate is transported into synaptic vesicles mostly by the vesicular glutamate transporter 1 (VGLT1; SLC17A7). Glutamate itself is the source for the synthesis of GABA: Glutamic acid decarboxylases (GAD1 and GAD2) convert the main excitatory neurotransmitter glutamate into the main inhibitory neurotransmitter GABA. GABA is then carried into the synaptic vesicle by the vesicular inhibitory amino acid transporter (VIAAT; SLC32).



Synaptic Transmission, Fig. 1 Glutamatergic synaptic transmission. The presynaptic terminal contains voltage-dependent Na^+ and Ca^{2+} channels, docked vesicles, a vesicle during exocytosis, the Ca^{2+} sensor synaptotagmin, and the SNARE proteins which are involved in the exocytosis. The vesicular glutamate transporter VGLT1, a group of plasmalemmal glutamate transporters (EAAT3–EAAT5), and a group of presynaptic GPCRs ($mGluR_{2-4}$) are also shown. The postsynaptic cell contains two ligand-gated glutamate receptors (AMPA-R and NMDA-R) and a group of GPRCs ($mGluR_{1-8}$). The axon terminal and the postsynaptic density are held together by the synaptic cell adhesion molecule SynCAM

Acetylcholine

Cholinergic axon terminals contain choline acetyltransferase, which acetylates choline to acetylcholine. In these axon terminals, the synaptic vesicles are filled with acetylcholine by the vesicular acetylcholine transporter (VAChT, SLC18A3).

Monoamines

Dopaminergic axon terminals contain tyrosine hydroxylase, which converts tyrosine into levodopa, and aromatic l-amino acid decarboxylase, which decarboxylates levodopa to dopamine. Noradrenergic terminals contain in addition dopamine β-hydroxylase, which oxidizes dopamine to noradrenaline. The synthesis of serotonin starts with the essential amino acid tryptophan. Tryptophan is at first hydroxylated by tryptophan hydroxylase and then decarboxylated by aromatic l-amino acid decarboxylase to yield serotonin. The vesicular monoamine transporter 2 (VMAT2, SLC18A2) is responsible for the vesicular transport of dopamine, noradrenaline, and serotonin.

Neuropeptides

In contrast to the small transmitter molecules, the neuropeptides (e.g., opioid peptides) are synthesized in the rough endoplasmic reticulum of the neuronal perikarya. They are enclosed in vesicles in the Golgi apparatus. The vesicles travel down to the terminals by axonal transport.

Transmitter Release

Neurotransmitters are released from the active zone of the presynaptic axon terminal by the following cascade: arrival of the action potential at the terminal, depolarization, opening of voltage-sensitive Ca^{2+} channels (VGCCs), and exocytosis (Fig. 1) (Südhof 2013). The nerve action potential is mainly carried by Na^{+} entry through voltage-dependent Na^{+} channels. Axon terminal voltage-sensitive Ca^{2+} channels are mainly of the N and P/Q types.

Docking and Priming

During the first step, which is called "docking," complete synaptic vesicles are positioned in the vicinity of the presynaptic plasma membrane (Fig. 1). During the next "priming" step, docked synaptic vesicles are prepared for "fusion" with the presynaptic plasma membrane. Calcium influx through N- and P/Q-type Ca^{2+} channels triggers the vesicle fusion. The calcium channels are clustered in the close vicinity of the Ca^{2+} sensor synaptotagmin in the membrane of the synaptic vesicle. The activation of only a few of calcium channels is sufficient to trigger vesicle fusion.

Exocytosis

Exocytosis consists of the fusion of the vesicle membrane with the plasmalemma, the opening of a pore in the fused membranes, and outward diffusion of the neurotransmitter. Three proteins, the so-called SNARE proteins, are essential in exocytosis. One, synaptobrevin, is a protein of the vesicle membrane; the other two, syntaxin and SNAP-25 (synaptosome-associated protein of 25 kD), are proteins of the plasmalemma (Fig. 1). Upon an increase in cytoplasmic Ca^{2+} from about 0.1 to about 100 μM, the vesicular synaptobrevin grabs hold of the plasmalemmal SNAREs, and the three proteins intertwine into a complex that then tightens like a zipper. This "zippering" of the SNAREs pulls the vesicle and plasma membranes together so that the lipid bilayers merge. Ca^{2+} channels and SNAREs lie closely together. Syntaxin and SNAP-25 in fact interact physically with the α1 subunit of N- and P/Q-type Ca^{2+} channels. The close neighborhood permits the fastness of exocytosis; the time from the arrival of the action potential to pore formation is only 100 μs or less. This fast presynaptic component of synaptic transmission contributes to the overall high speed of synaptic transmission: The time period between the appearance of the presynaptic action potential and the postsynaptic response is frequently less than 1 ms (synaptic delay).

After exocytosis, the synaptic vesicle membrane with its lipids and proteins is recycled, either by immediate re-filling with transmitter or by passing through a vesicle resting pool deeper inside the axon terminal.

Stochastic Release

Action potential-elicited neurotransmitter exocytosis is a probabilistic process (Pulido and Marty 2017). Only a fraction of the action potentials elicits transmitter release: release probability varies greatly among synapse types; probability values of 5–20% are not uncommon. Moreover, even in the case of regular presynaptic action potentials, release events are not regular but occur at variable intervals. The probability of release can be modulated by presynaptic receptors in the axon terminal membrane (Fig. 1). Through these receptors, transmitter substances from neighboring neurones and hormones can increase or reduce the release probability. Axon terminals thus integrate the release command of the action potential and various modulatory chemical messages from the neighborhood to release an appropriate amount of transmitter.

Postsynaptic Receptor Activation

The neurotransmitter released from the presynaptic active zone diffuses through the synaptic cleft to the postsynaptic neuron. The postsynaptic receptors are mostly localized in the cell membrane of the dendrites or dendritic spines, in a region called postsynaptic density (Fig. 1). The presynaptic active zone and the postsynaptic density are closely held together with the help of cell adhesion molecules anchored in the axon terminal and the postsynaptic neuron.

Of the several classes of receptors for endogenous chemical signals (Hofmann 2017), two are used as postsynaptic receptors in synaptic

transmission: ligand-gated ion channels (LGICs) and G-Protein-Coupled Receptors (GPCRs). Due to the large number of transmitters and the existence of several receptor types for almost all, postsynaptic receptor activation is the most diversified step of synaptic transmission. Table 1 shows selected neurotransmitter receptors.

Ligand-Gated Ion Channels (LGIC)

LGICs are hetero- or homo-oligomeric proteins consisting of three, four, or five peptide chains, of which each spans the membrane several times (Alexander et al. 2019a). The three to five subunits surround a pore, which in the absence of transmitter is closed. The nicotinic receptor for acetylcholine, the $GABA_A$-receptor, the glycine receptor, and the 5-HT_3-receptor for serotonin are pentamers. The ionotropic glutamate receptors (iGluRs) are tetramers. The P2X-receptors for ATP are trimers. When transmitter is bound to a ligand-gated ion channel receptor, the open probability of the pore is increased. Ions may then

Synaptic Transmission, Table 1 Selected neurotransmitter receptors

Physiological transmitter	Receptor[a]	Effector mechanisms	Pharmacological agonists	Pharmacological antagonists
Glutamate	AMPA[b] (LGIC)	Na^+ and K^+ conductance ↑	AMPA	
	kainate (LGIC)	Na^+ and K^+ conductance ↑	kainate	
	NMDA[b] (LGIC)	Na^+, K^+, and Ca^{2+} conductance ↑	NMDA	NMDA: ketamine, amantadine
	[c]$mGluR_{1,5}$ (GPCR)	$G\alpha_{q/11}$		
	$mGluR_{2,3}$ -(GPCR)	$G\alpha_{i/o}$		
	$mGluR_{4,6,7,8}$ (GPCR)	$G\alpha_{i/o}$		
GABA	$GABA_A$ (LGIC)	Cl^- conductance ↑	muscimol	bicuculline
	$GABA_{B(1a,1b,2)}$ (GPCR)	$G\alpha_{i/o}$	baclofen	
Glycine	Glycine (LGIC)	Cl^- conductance ↑		strychnine
Acetylcholine	Nicotinic (LGIC)	Na^+, K^+, and Ca^{2+} conductance ↑	nicotine, suxamethonium	tubocurarine, pancuronium
	Muscarinic M_{1-5} (GPCR)	M_1, M_3, M_5: $G\alpha_{q/11}$	muscarine, carbachol	atropine, butylscopolamine, tiotropium, trospium
		M_2, M_4: $G\alpha_{i/o}$		
Dopamine	D_{1-5} (GPCR)	D_1, D_5: $G\alpha_s$	D_{2-3}: bromocriptine	D_{2-3}: haloperidol, amisulpride, risperidone
		D_2, D_3, D_4: $G\alpha_{i/o}$	D_{2-4}: pramipexole	
Noradrenaline	$\alpha_{1A,B,D}$ (GPCR)	$G\alpha_{q/11}$	phenylephrine	prazosin, tamsulosin
	α_{2A-C} (GPCR)	$G\alpha_{i/o}$	clonidine dexmedetomidine	yohimbine, mirtazapine
	β_{1-3} (GPCR)	$G\alpha_s$	isoprenaline	propranolol, metoprolol
			salbutamol	
			mirabegron	
Serotonin	5-HT_{1A-F}	$G\alpha_{i/o}$	5-$HT_{1B,D}$: sumatriptan, ergotamine	
	5-HT_{2A-C} (GPCR)	$G\alpha_{q/11}$		
	5-HT_3 (LGIC)	Na^+ and K^+ conductance ↑		ondansetron
Opioid peptides	μ, δ, κ (GPCR)	$G\alpha_{i/o}$	μ: morphine	naloxone
			μ: fentanyl	

[a]*LGIC* ligand-gated ion channel, *GPCR* G protein-coupled receptor
[b]*AMPA* α-**am**ino-3-hydroxy-5-methyl-4-isoxazole **p**ropionic **a**cid, *NMDA* ***N*-m**ethyl-**D**-**a**spartic acid
[c]*mGluR* metabotropic glutamate receptor

enter or leave the cell, but a selectivity filter in the channel lets only certain ions pass.

LGIC/Glutamate Receptors

The AMPA and kainate receptors for glutamate are cation channels; they are permeable for Na^+ and K^+ (some AMPA receptors also for Ca^{2+}) (Table 1) (Henley and Wilkinson 2016). When they open, the major consequence is a sudden entry of Na^+, and the ensuing depolarization is called excitatory postsynaptic potential (EPSP; Fig. 1). The NMDA receptor is also activated by glutamate, but glycine is needed as a co-activator. The NMDA receptor is blocked by Mg^{2+} at resting membrane potentials, and this blockade is relieved by depolarization. The NMDA receptor ion channel is permeable for Na^+, K^+, and Ca^{2+}. Ca^{2+} entering into the postsynaptic neuron via the NMDA receptor can activate intracellular event cascades involving calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII).

LGIC/$GABA_A$- and Glycine Receptors

The $GABA_A$-receptor and the glycine receptor are Cl^- channels (Table 1). When they open at a resting membrane potential of about −60 mV, the consequence is an entry of Cl^- which elicits hyperpolarization. This hyperpolarization is called inhibitory postsynaptic potential (IPSP).

All these postsynaptic events last only for a few milliseconds; synaptic transmission through LGICs is fast. When the postsynaptic cell membrane is sufficiently depolarized, voltage-dependent Na^+ channels open, and an action potential is generated.

G Protein-Coupled Receptors (GPCRs)

GPCRs are proteins that span the postsynaptic cell membrane seven times (heptahelical receptors) (Alexander et al. 2019b). Small ligands are usually bound within a pocket formed by the seven transmembrane helices. The large neuropeptides bind to the extracellular domains. When the receptors are activated, they interact with the appropriate G proteins that are bound to the inner surface of the cell membrane. The G proteins then pass the information on to various effectors (Fig. 1).

GPCR/$G\alpha_{i/o}$ Coupling

The $mGluR_{2,3,4,6,7,8}$ glutamate receptors; $GABA_B$ receptors; M_2 and M_4 muscarinic receptors; D_2, D_3. and D_4 dopamine receptors; α_2-adrenoceptors; $5\text{-}HT_{1A–F}$ serotonin receptors; and μ, δ, and κ opioid receptors all couple to $G\alpha_{i/o}$ proteins. Via G protein Gβγ subunits, G protein-gated inwardly rectifying potassium (GIRK) channels are activated, and the membrane potential becomes more negative. Activation of $G\alpha_{i/o}$ protein-coupled receptors also lowers the open probability of N- and P/Q-type Ca^{2+} channels. The cytoplasmic level of the second messenger cyclic AMP (cAMP) is lowered as well: less cAMP means less activation of the hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channel family. Neurotransmitters acting on postsynaptic $G\alpha_{i/o}$-coupled receptors usually slow the firing rate of postsynaptic neurons by opening GIRK channels but also by attenuating the cAMP-dependent activation of HCN channels. Presynaptically localized $G\alpha_{i/o}$-coupled receptors inhibit the transmitter release from the axon terminal.

GPCR/$G\alpha_{q/11}$ and $G\alpha_s$ Coupling

The $mGluR_{1,5}$ glutamate receptors; the M_1, M_3, and M_5 muscarinic receptors; and the α_1-adrenoceptors couple to $G\alpha_{q/11}$ proteins and thereby increase the cytoplasmic levels of the messengers inositol trisphosphate, diacylglycerol, and Ca^{2+}. Finally, the D_1 and D_5 dopamine receptors and the β-adrenoceptors couple to $G\alpha_s$ and thereby increase the cytoplasmic level of cyclic AMP.

These cascades of reactions need time in the range of seconds: synaptic transmission through GPCRs is slow. All further postsynaptic changes depend on the type of the postsynaptic cell. For example, activation of β_2-adrenoceptors causes in the heart an increase of the rate and force of contraction; in skeletal muscle glycogenolysis and tremor; in smooth muscle relaxation; in bronchial glands secretion; and in sympathetic nerve terminals, an increase in transmitter release.

Termination of Synaptic Transmission

Once released, neurotransmitters are inactivated by diffusion into the neighboring extracellular space, combined with one of two specific

pathways: either extracellular degradation by enzymes that face the extracellular space or uptake into cells.

Extracellular Degradation of Transmitters

Extracellular degradation removes acetylcholine, the neuropeptides, and ATP. Acetylcholine is rapidly hydrolyzed to choline and acetate by acetylcholinesterase (Rotundo 2017). The enzyme is localized in the presynaptic and postsynaptic cell membrane and in the extracellular matrix and splits about 10,000 molecules of acetylcholine per second. High concentrations of butyrylcholinesterase are found in the brain and many peripheral tissues. Although butyrylcholinesterase is also capable of cleaving acetylcholine, under physiological conditions its contribution to the elimination of acetylcholine released by neurons is minimal.

Elimination of Transmitters by Transporters

Glutamate, GABA, glycine, dopamine, noradrenaline, and serotonin are taken up into adjacent cells by plasmalemmal neurotransmitter transporters (Fig. 1). The transporters for GABA, glycine, dopamine, noradrenaline, and serotonin belong to the SLC6 transporter family (Kristensen et al. 2011). These transporters possess 12 transmembrane domains and use the electrochemical driving force of Na^+ across the neuronal membrane for moving the neurotransmitters against their concentration gradients into the neurons. The GABA transporter GAT1 is responsible for the re-uptake of GABA from the synaptic cleft into the axon terminal. GAT3 and BGT1 transfer GABA into adjacent glial cells. Glycine is transported into glycinergic axon terminals and glial cells by GLYT2 and GLYT1, respectively. The dopamine (DAT), noradrenaline (NAT), and serotonin transporters (SET) are located almost exclusively in the dopaminergic, noradrenergic, and serotoninergic terminals, respectively. In all these cases, cellular uptake means re-uptake into the presynaptic neurone. Re-uptake by the plasmalemmal transporter may be followed by vesicular re-uptake, an economical way of inactivation, reminiscent of the recycling of the storage vesicle membrane.

The glutamate transporters belong to the SLC1 transporter family, possess 12 transmembrane segments, and also use the transmembrane electrochemical gradient of Na^+ to pay for the transport work. Both neuronal (EAAT3, EAAT4, and EAAT5) and glial (EAAT1 and EAA2) transporters can remove glutamate from the synaptic cleft (Fig. 1).

Modulation of Synaptic Transmission

Synaptic plasticity. The strength of synaptic transmission (size of the postsynaptic response to a presynaptic action potential) is often modulated under physiological conditions, and this process is called "synaptic plasticity." The strength can be increased (potentiation) or decreased (depression). The duration of the change in synaptic strength can be short term (under 1 min) or long term (longer than ~30 min, up to life-long). Synaptic plasticity is important for memory and learning.

The mechanisms of synaptic plasticity are manifold. Transmitter release can be rapidly changed by altering the number of docked vesicles in the active zone. The number of postsynaptic receptors within the postsynaptic density can also be quickly changed. Longer time periods are necessary for the synthesis of new synaptic proteins and for morphological changes of the postsynaptic dendrites and dendritic spines. A classical form of long-term potentiation (LTP) is the NMDA receptor-dependent associative LTP of glutamatergic synaptic transmission (Lüscher and Malenka 2012). In this case, the presynaptic axon and the postsynaptic neuron are simultaneously depolarized (associative inputs). Glutamate released from the axon terminal activates postsynaptic AMPA and NMDA receptors. The postsynaptic depolarization enables the NMDA receptor to conduct Ca^{2+}. Ca^{2+} triggers intracellular effectors, and as a result, more AMPA receptors are moved into the postsynaptic density. Thus, the associative stimulus elicits an LTP of the AMPA receptor-mediated glutamatergic synaptic transmission.

Presynaptic Receptors

Transmitter release can be modulated also by activation of presynaptic receptors in the axon terminals. The presynaptic receptors are often autoreceptors (i.e., they respond to the transmitter which was released from the axon terminal) and

mostly $G\alpha_{i/o}$ protein-coupled receptors. Thus, presynaptic autoreceptors mediate negative feedback to limit further transmitter release – this is a presynaptically expressed short-term homeostatic synaptic plasticity. α_2-Adrenoceptors on noradrenergic axon terminals are prototypic presynaptic autoreceptors and were much studied (Starke 2001).

Retrograde Signaling

In the case of retrograde signaling, synaptic information flows retrogradely, i.e., from the postsynaptic neuron to the presynaptic axon terminal. Endocannabinoid-mediated retrograde signaling is probably the most ubiquitous form of retrograde signaling. The endocannabinoid 2-arachidonoylglycerol is released from postsynaptic neurons upon depolarization and subsequent calcium influx or after activation of $G\alpha_{q/11}$ protein-coupled receptors (Araque et al. 2017). After crossing the synaptic cleft, 2-arachidonoylglycerol activates presynaptic CB_1 cannabinoid receptors, which leads to a decrease of transmitter release from the axon terminal. This is an endocannabinoid-mediated short-term synaptic plasticity.

Clinical Pharmacology: Drugs Interfering with Synaptic Transmission

Transmitter Production and Vesicular Storage

Levodopa is administered as the precursor of dopamine to compensate for the loss of dopaminergic neurones in Parkinson's disease (see Chapter "Dopamine System"). After passing the blood-brain barrier, levodopa is converted into dopamine by aromatic l-amino acid decarboxylase. Reserpine blocks VMAT2; thus it depletes noradrenaline from the sympathetic axon terminals, and it was used in the past to treat hypertension. Because reserpine simultaneously empties noradrenergic, dopaminergic, and serotoninergic synaptic vesicles in the brain, many central nervous system side effects occur. A major component of the anticonvulsive effect of valproate is enhancement of GABAergic synaptic transmission in the brain by enhancing GABA synthesis in GABAergic axon terminals.

Transmitter Release

A large number of natural poisons inhibit transmitter release from axon terminals. Tetrodotoxin, produced by the puffer fish, occludes voltage-gated Na^+ channels by binding to the external side of the channel pore: as a consequence, the axon terminal is no longer depolarized. The cone snail *Conus geographus* produces 9-conotoxin GVIA, which blocks N-type Ca^{2+} channels. P-/Q-type Ca^{2+} channels are selectively blocked by the funnel-web spider venom 9-agatoxin IVA.

The most ingenious exocytosis toxins, however, come from the anaerobic bacteria *Clostridium botulinum* and *Clostridium tetani*. The former produces seven botulinum neurotoxins (BoNTs) A–G; the latter produces tetanus neurotoxin (TeNT) (Dong et al. 2019). All eight toxins consist of a heavy (H) chain and a light (L) chain that are associated by an interchain S–S bond. The L-chains enter the cytosol of axon terminals and then specifically split one of the three core proteins of exocytosis: synaptobrevin, syntaxin, or SNAP-25 (SNARE proteins; Fig. 1). BoNT L-chains mainly enter peripheral cholinergic terminals: the inhibition of acetylcholine release is followed by flaccid paralysis, the main symptom of botulism. The TeNT L-chain mainly enters cerebral and spinal cord GABAergic and glycinergic terminals: the inhibition of GABA and glycine release is followed by spastic paralysis, the main symptom of tetanus. BoNT and TeNT are the most potent toxic substances known, able to kill vertebrates at a dose of 0.1 to 1 ng/kg body weight. Due to its ability to inhibit acetylcholine release from cholinergic neurons, BoNT is increasingly used in therapy, for example, for the treatment of muscle spasticity, migraine, axillary hyperhydrosis, and overactive bladder. Undoubtedly, BoNT is most frequently used to alleviate wrinkles on the face – a cosmetic indication (in this setting BoNT is commonly called "Botox").

Mirtazapine is one of the most often-used antidepressant drug: it increases the release of noradrenaline and serotonin from axon terminals by blocking presynaptic α_2 adrenoceptors. The anticonvulsive drug levetiracetam lowers glutamate release by interfering with the docking and fusion

of synaptic vesicles after a primary interaction with the vesicle protein SV2.

Postsynaptic Receptor Activation

The most diversified step of synaptic transmission is also the target of the greatest variety of drugs. Some are included in Table 1. Many drugs and poisons stem from plants, for example, muscimol, bicuculline, strychnine, nicotine, tubocurarine, muscarine, atropine, scopolamine, and morphine.

The therapeutic impact of drugs acting at transmitter receptors is enormous. All volatile (isoflurane) and intravenous (propofol) anaesthetics and also ethanol act primarily on cerebral LGICs, above all $GABA_A$-receptors. All neuromuscular blocking agents are agonists (suxamethonium) or antagonists (pancuronium) at the skeletal muscle nicotinic receptor. There would be no surgery worth mentioning without these drug actions on neurotransmitter receptors. The anti-Parkinson drug pramipexole activates D_2 and D_3 dopamine receptors. Another drug used in Parkinson's disease, amantadine, is an antagonist at the NMDA glutamate receptor. Morphine and related opioid agonists are the most effective analgesics. The benzodiazepines (diazepam, midazolam) are positive allosteric modulators of the $GABA_A$-receptors and possess sedative, anticonvulsive, and anxiolytic actions. Neuroleptics such as haloperidol and olanzapine block D_2 dopamine receptors and are standard drugs to treat schizophrenia.

β_1 adrenoceptor antagonists such as metoprolol are mainly used in cardiology and belong to the most frequently prescribed drugs. The α_1 adrenoceptor antagonist tamsulosin is a first-line drug for the treatment of benign prostatic hyperplasia. Antagonists of muscarinic acetylcholine receptors are used to treat asthma and COPD (ipratropium, tiotropium), overactive bladder (trospium), and biliary and renal colic (butylscopolamine).

Transmitter Inactivation

The cholinesterase inhibitors are the classical drugs that interfere with transmitter inactivation. The prototype was the plant poison physostigmine, and derivatives of physostigmine are used to treat myasthenia gravis. The highly toxic nerve gases such as sarin are also cholinesterase inhibitors. Monoamine oxidases (MAOs) metabolize noradrenaline, dopamine, and serotonin. MAO inhibitors are used in the treatment of depression and Parkinson's disease (tranylcypromine and rasagiline, respectively). The GABA transporter is blocked by tiagabine, an antiepileptic that owes its effect to the ensuing increase of the concentration of GABA in the synaptic cleft. Cocaine is abused because it blocks the dopamine and serotonin transporters and, hence, enhances dopaminergic and serotoninergic transmission in the mesolimbic "reward system." Inhibitors of the serotonin (fluoxetine, citalopram, amitriptyline) and noradrenaline (amitriptyline) transporters are the main antidepressant drugs.

Cross-References

- ▶ Antidepressant Drugs
- ▶ Botulinum Neurotoxins
- ▶ Cholinesterases
- ▶ Dopamine System
- ▶ G-Protein-Coupled Receptors
- ▶ Glycine Receptors
- ▶ Nicotinic Receptors

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Synthetic Lethality

► DNA Damage Response

Syntocinon

► Oxytocin

Systemic Inflammation

► Sepsis