Two Birds with a Stone: Molecular Cancer Therapy Targeting Signal Transduction and DNA Repair Pathways

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Abstract The hallmarks of cancer cells are a higher proliferative activity and an aberrant genotype with respect to normal cells. These features can be exploited for the development of selective chemotherapeutic treatments against cancer. In particular, the connections among signal transduction pathways, cell cycle checkpoints and DNA replication and repair have the potential to provide new venues for the treatment of cancer. Here, we will review how the differences existing between normal and tumour cells, with respect to control of cell proliferation and maintenance of the genetic stability, can be exploited in cancer chemotherapy.

Keywords DNA repair · DNA polymerase · Cancer · Tyrosine kinase · Signal transduction · Anticancer drugsanticancer drugs

Abbreviations

BIR	Break induced replication
CK2	Casein kinase II
CML	Chronic myeloid leukemia
DDR	DNA damage response
DSBs	Double strand breaks
GISTs	Gastrointestinal stromal tumours
HER-2	Human epidermal growth factor receptor 2
MMEJ	Microhomology-mediated end joining
NRTKs	Non receptor tyrosine kinases
NSCLC	Non-small cell lung cancer
PDGFR	Platelet-derived growth factor receptor
PTEN	Phosphatase and tensin homology protein
SFK	Src family kinase

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SH2	Src homology-2
SH3	Src homology-3
SSA	Single-strand annealing
SSBs	Single strand-breaks
STKs	Serine-threonine kinases
TKs	Tyrosine kinases
VEGFR	Vascular endothelial growth factor receptor

Introduction

Tumour cells are characterized by a higher proliferative activity with respect to the surrounding cells. This hallmark of cancer cells has been regarded since the beginning as a feature to be exploited for the development of selective chemotherapeutic treatments against cancer. Indeed, anticancer chemotherapy can be regarded as the science of selective toxicity, since it is aimed at reducing the proliferation of cancer cells with minimal perturbation of the homeostasis of normal cells.

In order to achieve selectivity, however, a first requirement to be fulfilled is the identification of a suitable target. Ideally, such a molecular entity should play an essential role in cancer cells, while being dispensable for the normal life of healthy cells. Once a suitable target is identified, small molecules need to be developed which selectively suppress that particular molecular function, without interfering with other similar proteins eventually present in the cells.

In practice, neither of these two goals has been fully achieved in contemporary anticancer chemotherapy. Classical anticancer drugs, in fact, target proteins which are involved in the proliferation of both normal and diseased cells. Selectivity is achieved on the basis that in adult organisms only a small subset of cells have proliferative indexes similar to cancer ones, thus administering the correct doses of drugs for a limited period of time, may achieve suppression of cancer growth without making too much damage to the normal cells.

In recent years, thanks to the advancement of our understanding of the physiology of cancer cells, it was realized that tumourigenesis is almost invariably driven by aberrations in various signal transduction and DNA repair pathways. As a result, cancer cells are dependent, for their survival, on a narrower complement of molecular functions than normal cells. Fostered by these findings, novel targets have been identified, such as tyrosine kinases and DNA repair enzymes. Selective inhibitors of these new targets have been developed and have already entered the clinics. However, after a few years, also these new approaches did not completely fulfill the expectations of the researchers. In many instances, drug resistance readily developed and, thus, reducing the efficacy of the drugs. In addition, alternative pathways, sometimes tumour-specific, can be activated in cancer cells to surrogate for the functions inhibited by the drugs.

As a result, it is now clear that a shift of paradigm is required to develop a new generation of anticancer drugs. Genome-wide analyses of cancer cells proteomes and transcriptomes have provided the experimental evidence for the existence of truly different molecular phenotypes among different tumour types. Thus, cell proliferation cannot be considered anymore, according to the simplistic view of the past, as the hallmark of all cancers since the molecular pathways leading to uncontrolled proliferation are very diverse.

A more fruitful approach should be based on the understanding of the links connecting signal transduction pathways to cell cycle checkpoints and DNA replication and repair. Proliferative signals, which are transduced by tyrosine kinases, beside inducing DNA replication, also activate those pathways that are required to maintain a level of genomic stability compatible with cell survival, including DNA repair. These pathways are the same that also allow tumour cells to cope with the DNA damage induced by classic anticancer agents such as etoposide or cis-Pt. At the same time, tumours are very often defective in one or more DNA repair pathways, thus depending on the remaining ones for their survival. Thus, a strategy aimed to specifically target both signal transduction and DNA damage tolerance pathways may prove to be effective on a large fraction of tumours. An additional benefit of such a combination targeted chemotherapy could be to reduce the emergence of drug resistance. In fact, the genetic barrier required to develop at least one independent mutation for each target will be higher than in the case of monotherapy regimens. To date, several signal-transducing kinases are being regarded as attractive targets for selective cancer chemotherapy. In addition, the realization that in human cells there are at least 15 different DNA polymerases playing non-overlapping roles in DNA replication and repair, has provided entirely new venues for the development of novel cancer drugs.

Targeting Signal Transducing Pathways

Phosphorylation is a fundamental mechanism used in transduction pathways to propagate the signal to final effectors. The reaction consists in the transfer of the —phosphate from ATP to amino acidic residues in specific peptide substrates. The enzymes responsible for this reaction are tyrosine kinases (TKs) and serine-threonine kinases (STKs). These enzymes regulate multiple aspects of cellular metabolism, determining differentiation, adhesion, motility, genome stability, cell growth and death.

Receptor Tyrosine Kinases (RTKs) are single transmembrane domain receptors resident in the plasma membrane with high affinity for ligands like growth factors, cytokines or hormones. Unlike RTKs, non receptor tyrosine kinases (NRTKs) lack both extracellular and transmembrane domains and can be found free in the cytosol as well as in the nucleus, or linked to the inner cell membranes by myristoylation or palmitoylation modifications. These enzymes harbour protein–protein interaction domains like Src homology-2 (SH2), Src homology-3 (SH3)

and pleckstrin homology domains by which NRTKs interact with substrates or regulatory factors.

STKs include a large number of kinases whose activity can be regulated by numerous chemical signals, including DNA damage, cAMP/cGMP, diacylglycerol and Ca2+/calmodulin. Their activity is crucial in the genome stability and its maintenance. Given their roles within the cell, their overexpression or deregulation are linked to the onset, progression and malignancy in a wide range of cancers. Moreover, the deregulation of one or more specific kinases appears to have a positive effect on particular cancer cells survival. Therefore, the specific inhibition of such kinases leads to cancer cell death without impairing the survival of healthy cells. For these reasons, kinases represent ideal candidates for cancer targeted therapy.

BCR-Abl as the Prototype of Molecular Targeted Chemotherapy

The classic example of a successful therapy targeted to a protein kinase is the inhibition of the Abl kinase in leukemias. NRTKs Abl1 and Abl2 are members of the Abl family of kinases, involved in actin remodelling, cell adhesion and motility, DNA damage response and microbial pathogen response [1]. Equally to the Src Family Kinase (SFK) members, Abl proteins can exist either in an active (open), or inactive (closed) form. The shift from open to close conformation is regulated through self interaction between the regulatory domains SH2 and SH3 and the C-terminal part of the protein. *ABL* genes are constitutively activated by chromosome translocations in various haematopoietic malignancies. Chronic myeloid leukemia (CML) is characterized in almost all cases by a t(9q34;22q11) translocation that fuses the Bcr (breakpoint cluster region) and ABL1 genomic regions. The Bcr-Abl1 fusion gene product (p210) has a constitutive tyrosine kinase activity that leads to the activation of the downstream pathways of Abl, conferring to the haematopoietic cell a tumoral phenotype [2]. A similar translocation occurs in three to five percent of childhood [3] and 20–30 % of adult acute lymphoblastic leukemia (ALL) cases. Additional ABL1 fusion variants like NUP214-ABL1, EML1-ABL1 and ETV6-ABL1 [4–6] have been reported in some other leukemias, such as acute myeloid leukemias (AMLs) [7]. In all of these cases, the cell transformation activity by ABL fusion proteins is inextricably tied to their tyrosine kinase activity. In May 2001, the FDA approved Imatinib as the first-line treatment for CML. Imatinib is a selective TK inhibitor which competes with ATP in binding to the Bcr-Abl protein kinase. In particular, the drug occupies a part of the ATP-binding pocket of the enzyme and stabilizes the inactive, non-ATP-binding form of Bcr-Abl [8]. Imatinib has shown excellent activity against CML, inducing apoptosis in leukemia cells; its introduction in therapy greatly improved the outcome of CML patients with complete haematological remission

in more than 90 % of previously treated patients who are resistant to interferon treatment [9, 10].

Beyond Imatinib: New Generation TK Inhibitors

After Imatinib, many other small-molecule inhibitors specifically targeting different kinases have been approved for the treatment of different cancers or are in clinical trial (Table 1). Erlotinib and Gefitinib are small-molecule inhibitors targeting the Epidermal Growth Factor Receptor (EGFR) Family that are approved in breast and non–small cell lung cancers therapy. EGFR is the cell-surface receptor of extracellular protein ligands of the epidermal growth factor family (EGF-family) members. As for other RTKs, binding of the ligand stimulates the dimerization of EGFR resulting in autophosphorylation and, consequently, in the full activation of the kinase domain [11]. In non–small cell lung cancer (NSCLC), which accounts for approximately 85 % of lung cancer cases, EGFR is overexpressed or hyper activated through somatic gain-of-function mutations in exons encoding the EGFR tyrosine kinase domain (in-frame deletions in exon 19 or L858R substitution) [12, 13].

Table 1 Tyrosine kinase inhibitors in anticancer therapy

Inhibitor	Target
Small molecule inhibitors	
Axitinib	Multiple targets
Bosutinib	Bcr-Abl/SRC
Crizotinib	ALK/MET
Dastinib	Multiple targets
Erlotinib	EGFR
Gefitinib	EGFR
Imatinib	Bcr-Abl
Lapatinib	ErbB1/ErbB2
Nilotinib	Bcr-Abl
Pazopanib	VEGFR2/PDGFR/KIT
Pegaptanib	VEGFR
Ruxolitinib	JAX
Sorafenib	Multiple targets
Sunitinib	Multiple targets
Vandetanib	RET/VEGFR/EGFR
Vemurafenib	BRAF
Monoclonal antibodies	
Bevacizumab	VEGFR
Cetuximab	ErbB1
Panitumumab	EGFR
Ranibizumab	VEGFR
Trastuzumab	Erb2

Erlotinib and Gefitinib affect the EGFR kinase activity by acting as ATP competing molecules, binding in a reversible fashion to the ATP-binding site of the receptor. Like EGFR, Human Epidermal growth factor Receptor 2 (HER-2) is a member of the EGFR family. HER-2 is a RTK normally involved in signal transduction pathways leading to cell growth and differentiation, migration and apoptosis. It is considered an orphan receptor because none of the Epidermal Growth Factor (EGF) ligands are able to activate it. On the other hand, HER-2 is the preferential dimerization partner of all other members of the EGFR family. HER-2 amplification or overexpression in breast cancer was found to be correlated with aggressive tumour growth and poor clinical prognosis, rendering HER-2 an ideal candidate for chemotherapy [14]. Lapatinib is a selective, potent, small-molecule inhibitor of HER1 and HER-2 that is approved in combination with Capecitabine for the treatment of patients with advanced or metastatic breast tumour stages with overexpression of HER-2 [15, 16]. The binding mechanism resembles that one of Imatinib to Abl, hence, it binds to the inactive HER-2 form [17].

The Unmet Promises: Drug Resistance to TK Inhibitors

Despite the administration of these drugs that have greatly ameliorated the efficacy of the therapy, in many cases, the onset of drug resistance leads to treatment failure. Drug resistance can be achieved in different ways that are best described in ABL-related tumours. Indeed, the follow-up of CML patients receiving Imatinib showed that primary resistance (no response to Imatinib after the initial treatment) or secondary resistance (development of resistance after achieving an objective response) emerged in 31 % of the patients. In all cases, Imatinib resistance was characterized by the presence of active Bcr-Abl, rather than the activation by the cell of an alternative signaling pathway independent from Bcr-Abl [18] and, thus, indicating that the BCR-ABL signal transduction pathway is crucial to cancer cells survival. The mechanisms of acquired Imatinib resistance were due to BCR-ABL gene amplification or mutation events. In the first case, due to BCR-ABL gene amplification or overexpression, the intracellular Imatinib concentration is not high enough to inhibit all Bcr-Abl molecules in leukaemic cells [19, 20]. In the second case, BCR-ABL mutations emerged upon the selective treatment pressure. Up to now, nearly 100 ABL mutants have been described, some of these appearing with higher frequency than others: 15 single amino-acid substitutions account for more than 85 % of the reported mutations, and 66 % of reported cases occur specifically at seven sites only (G250, Y253, E255, T315, M351, F359, H396). Furthermore, different amino-acid substitutions can involve the same residue like F317C, F317L, and F317V, all showing reduced Imatinib sensitivity [21–23]. Since these mutations in the *BCR-ABL* fusion gene were observed only after Imatinib administration, it indicates that there must have been a low prevalence of mutant cells before the therapy. This idea is supported by the fact that these mutations do not provide any growth advantage in the absence of Imatinib, but are

selected specifically only upon drug exertion pressure [24]. Kinase activity is not abrogated by the mutations, although some mutants demonstrate lower enzymatic activity compared with the wild-type BCR-ABL. On the other hand, the kinase activity is enhanced by other type of mutation [25].

It has been shown that besides displaying inhibitory activity towards ABL, Imatinib is active against the RTKs KIT and PDGFRA [26]. KIT is a cytokine receptor expressed on the surface of hematopoietic stem cells and plays a role in cell survival, proliferation, and differentiation; PDGFRA is a member of the platelet-derived growth factor family implicated in mesenchymal cells proliferation. These RTKs are constitutively activated by gain-of-function mutations in most Gastrointestinal Stromal Tumours (GISTs) [27]. Experiments on human tumour cell lines, dependent on the KIT pathway, showed that Imatinib could block the activity of KIT in the GIST cells, arresting therefore proliferation and causing apoptosis. Currently, Imatinib is in phase II and III clinical trials for GIST treatment; the prognosis of GIST patients has dramatically improved after recruitment of Imatinib into the therapeutic arsenal (80 % clinical response rate). However, the mutation resistance issue arising during the Imatinib therapy significantly influences the clinical response also in the case of KIT. As for the BCR-ABL Imatinib resistance model, Imatinib efficacy is affected by the KIT mutations that directly block the drug binding or pose an energetic hindrance disfavouring the closed conformation of the kinase [28, 29].

Also in the case of Erlotinib and Gefitinib, targeting EGFR, after an initial response characterized by tumour regression and improvement in disease-related symptoms, most patients relapse. In response to Erlotinib or Gefitinib treatment, different resistance mechanisms can occur and one of the most frequent is the amplification of the *MET* proto-oncogene [30]. MET is another RTK (also known as Hepatocyte Growth Factor Receptor) and its amplification has also been observed in gastric and esophageal cancers [31, 32]. MET is involved in a pathway distinct from EGFR, and it is normally expressed by cells of epithelial origin where it promotes cell growth and motility [33]. In NSCLC cell lines whose Gefitinib resistance was obtained by continuous drug administration, a marked focal amplification within chromosome 7q31.1–7q33.3, containing the *MET* proto-oncogene, was observed. It is known that Gefitinib leads to the disruption of the signal cascade EGFR/ERBB3/PI3K/Akt [34, 35]. In Gefitinib resistant cell lines, MET amplification leads to PI3K/Akt signaling through ERBB3 activation, independently from EGFR; hence, MET inhibition restores sensitivity to Gefitinib. Resistance towards Erlotinib or Gefitinib can also occur as a consequence of mutations in the *K-RAS* gene, a GTPase of the Ras family, which is a downstream effector of EGFR, [36]. Similarly, treatment of HER-2 overexpressing cancers is not always effective because resistance to Lapatinib develops in some patients during prolonged exposure to the drug. It is known that resistance to Lapatinib in breast cancer arose through AXL overexpression. AXL is a RTK, closely related to MET, involved in cell proliferation and motility and, as the case of MET for NSCLC, its overexpression is associated with poor prognosis and increased invasiveness of several human cancers [37].

In order to avoid drug resistance, design of drugs with inhibitory properties against the mutant kinase forms is being actively pursued. It is the case of the ABL inhibitor Nilotinib, targeting Bcr-Abl with an increased potency (approximately 20-fold) when compared to Imatinib. Even if both drugs act in the same manner, hence, binding to the kinase domain of the inactive ABL form, Nilotinib binding is energetically more stable [38]. Nilotinib remains active towards all Imatinib resistant mutations lying in the activation domain (A-loop), as well as towards mutations spread across the entire Bcr-Abl kinase domain, including the P-loop. In fact, the clinical efficacy of Nilotinib was demonstrated in patients harbouring most Imatinib-resistant mutations, with the exception of Y253H, E255K/V, F359C/V and T315I BCR-ABL mutations. In particular, overcoming the Imatinib resistance mutation T315I, that confers resistance also towards Nilotinib with IC50 value 800-fold greater than against wild type Bcr-Abl, still constitutes a significant unmet medical need [39].

Overcoming Drug Resistance: The Dual Inhibitor Concept

A more innovative strategy to overcome drug resistance consists in the development of drugs able to inhibit not only the primary target kinase, but also other kinases that contribute to cancer survival. For instance, Src Family Kinases (SFKs) are downstream effectors of Bcr-Abl; Dasatinib, another second generation agent developed for the treatment of CML, besides inhibiting Bcr-Abl, has been shown to successfully inhibit also the SFKs Src, Lyn, Yes, and Lck [40]. In particular, Lyn and Hck are involved in signal transduction pathways downstream of Bcr-Abl. Thus, the activity of Dasatinib, especially towards LYN, contributes to the overall efficacy of this drug in the treatment of CML. Like Imatinib and Nilotinib, Dasatinib acts as a competitor of the ATP substrate, but differently from other drugs, it binds to ABL both in its active and inactive conformation [41]. Compared with Imatinib, Dasatinib has an approximately 300-fold increased potency in antiproliferative assays. Despite the fact that Dasatinib remains active towards the majority of Imatinib-resistance mutants, the mutations T315I/A and F317I still result in a completely resistant phenotype.

Many other drugs showing a dual activity against Abl and SFKs are already in different clinical trial phases and preliminary research investigations. For instance, Bafetinib and Bosutinib are other dual inhibitors of Abl and SFKs that have been tested in clinical trials for CML treatment. Bafetinib targets Abl and Lyn, showing limited inhibition against other SFKs [42]. Despite the fact that Bafetinib is effective in a heavily pretreated (Imatinib, Nilotinib, Dasatinib) population, it lacks any appreciable efficacy against T315I mutation and in blastic CML phases, or in Ph-positive ALL [43]. Another drug in phase III clinical trials for CML treatment, Bosutinib [44], shows similar limitations [39, 45].

The mutation T670I in KIT is selected in GIST tumours after prolonged Imatinib administration. T670 of KIT was identified as one of the key hydrogen

bonds for Imatinib binding [46], similarly to T315I in Bcr-Abl. In the same way, the EGFR T790M resistance mutation occurs after prolonged administration of Elrlotinib or Gefitinib in NSCLCs. Also, this mutation is in an analogous position to T315I in Abl and T670I in Kit [47].

In addition to small-molecule inhibitors, monoclonal antibodies (mAb) targeting RTK are also in use in clinical therapy (Table 1). Their use relies on the principle that the targeted receptors are expressed at higher levels on cancer cells than on healthy cells. Several mAbs are approved for cancer therapy or are in clinical trials. Targets of these mAbs are EGFR, HER2, VEGFR, MET, VEGFR2 and IGF1-R. The anti-cancer activity of a mAb is due to different mechanisms: the binding with the receptor can (1) prevent ligand-receptor interaction (2) promote receptor internalization (3) prevent receptor dimerization and activation and (4) induce apoptosis or immune response toward the target cells [48]. As for small-molecule inhibitors, primary and secondary resistance towards these drugs have emerged. Multiple studies showed that primary resistance can be conferred by activating mutations in KRAS, PIK3CA, BRAF or loss of PTEN expression. These mutations negatively correlate with the response to Cetuximab or Panitumumab, two EGFR-targeting mAbs [49]. Secondary resistance is due to (1) overexpression and aberrant phosphorylation of alternative RTK (2) expression of receptor variants (3) increased expression of the target receptor and (4) activation of alternative pathways [48]. To date, both point mutations in the target receptor or rearrangements in the corresponding genomic regions have been observed after mAb treatment.

Expanding the Dual-Inhibitor Concept: The Quest for Multi-Targeting TK Inhibitors

The drug development research field is still the problem of the unsatisfactory efficacy of to-date approved inhibitors towards the T315I substitution and analogue mutations in other kinases. Some encouraging data are now emerging in clinical trials with other kinase inhibitors. Sunitinib, approved as second line therapy of GIST and renal cell carcinoma (RCC), is a multi-targeted RTKs inhibitor active toward PDGFR, VEGFR, Kit, Ret, CSF-1R and Flt3 [50]. Contrary to Imatinib, it is also active towards several Imatinib-resistant KIT secondary mutations, including the T670I KIT mutant. In the same way, Sorafenib, a small-molecule inhibitor that targets the RTKs vascular endothelial growth factor receptor (VEGFR) and Platelet-derived growth factor receptor (PDGFR) and the STKs C-Raf and B-Raf [51], is also able to inhibit Kit mutation that provides resistance toward Imatinib and Dasatinib.

If the availability of inhibitors that target multiple kinases could result in a higher degree of transduction signal inhibition, and thus in higher efficacy on cancer treatment, it could also result in enhanced toxicity for healthy cells.

The identification of the hierarchical pattern of inhibition of a given compound against all the kinome is now emerging as an essential step in order to estimate the effect of a drug against a selected cancer. Recently, high-throughput screening approaches have been use by Anastassiadis et al. [52] and Davis et al. [53] with the aim to score the inhibition potency and selectivity of 72 and 128 known kinase inhibitors, respectively, against large panels of kinases (442 and 300, respectively). Traditionally, the discovery of kinase inhibitors starts from a high-throughput screening of small molecules with inhibitory properties targeting a kinase of interest. The selectivity of effective compounds is then evaluated against a panel of representative kinases. The same goal can be obtained by screening libraries of compounds against large panels of protein kinases, thus revealing the degree of selectivity of each compound. This method led to the identification of inhibitors that were unexpected for the kinase of interest, revealing multi-targeted inhibitors active on a number of kinase targets larger than predicted. For example, Sunitinib, known to target PDGFR, VEGFR, Kit, RET, CSF-1R and Flt3 [50], shows affinity for RET harboring gatekeeper mutations [RET (V804L/M)], which is resistant to the approved RET inhibitor Vandetanib. In the same way, PKC-412, a compound designed as a protein kinase C (PKC) inhibitor, was shown to be more active against the EGFR mutant T790M. These studies also revealed that compounds designed to target a specific kinase could show higher potency of inhibition against another, unrelated enzyme. For example, DMBI, designed as a Platelet-derived growth factor receptor (PDGFR) inhibitor, is an highly potent inhibitor of FLT3 and TrkC; SB202474, an inactive analog of the p38 MAP kinase inhibitor SB202190 [54], showed significant activity only against the haploid germ cell–specific nuclear protein kinase Haspin. These data open new opportunities for clinical use of drugs already tested for their pharmacological properties.

In order to improve the efficacy of target therapy and to fight/avoid the onset of resistance, a valid strategy should be the analysis of the signalling network surrounding a target kinase. The determination of a unique factor responsible for drug resistance is not possible in many tumours that show heterogeneous resistance due to partial contributions by multiple proteins. Network models paradigm conjecture that signalling pathways are made with no hierarchy and feedback loops and are redundant. This implicates that inhibiting a specific oncogene can lead to the rescue of the signaling by enrichment (gain of function, amplification, overexpression) of proteins that compose the web of interaction with the target. Astsaturov et al. [55] screened siRNA libraries targeting EGFR network in order to identify synthetic lethality with EGFR inhibitors. This approach consented to identify previously uncharacterized genes that can drive resistance modulating EGFR signaling or that can be considerate as concomitant target for the treatment of EGFR hyper activated cancers. For instance, in the case of the EGFR network, the inhibition of SFKs or Aurora A kinase enhances the effect on cancer viability of EGFR inhibitors. The application of this approach to other validated targets could greatly ameliorate the clinical strategy in other malignancies.

Why Targeting DNA Repair in Anticancer Chemotherapy?

RTKs and NRTKs are involved in transducing proliferative signals, hence, resulting in the activation of DNA replication and cell division. In turn, the high proliferative phenotype of tumour cells is very often accompanied by alterations in their genetic structure, leading to chromosomal aberrations and aneuploidy.

The genomic instability and the high mutation rate, typical features of human cancer cells, are mainly due to defects in DNA repair. In fact, very often at least one of the six major DNA repair pathways (mismatch repair MMR, base excision repair BER, nucleotide excision repair NER, homologous recombination HR, non-homologous end joining NHEJ and translesion synthesis TLS) is indeed defective in tumours. The mechanisms through which cancer cells respond to damaged DNA have important implications in the development of both the tumoral phenotype and the resistance to chemotherapy [56, 57].

Activation of the various DNA repair pathways during cell cycle progression depends on proper regulation of checkpoints, that are signalling cascades, often involving multiple kinases, leading to the activation of specific transcription factors. One of the main transcriptional effectors activated by DNA damage checkpoints is the protein p53. It is, thus, not surprising that *p53* is one of the most frequently mutated gene in human cancers due to its ability to halt cell growth and to modulate apoptosis after checkpoint activation when DNA damage and/or genotoxic stress occur [58].

Germline mutations of *p53* are associated with a disease called Li-Fraumeni Syndrome characterized by an increased risk of developing various cancers with an early age of onset [59]. Moreover, various clinical phenotypes in different types of cancer are associated with somatic *p53* mutations at specific residues [60]. The involvement of *p53* in DNA repair pathways has also a detrimental effect on chemosensitivity, helping cells to resist to DNA damage caused by therapy. An example is the well documented resistance against anthracyclines and mitomycin due to *p53* mutations in breast cancer as well as in haematological malignancies [61].

Polymorphism regarding *BER* genes such as *OGG1*, *APE1*, *MutYH* and *XRCC1* have been examined for their possible effect on cancer development [62]. As an example, a *DDR* gene strongly associated with cancer development is *MutYH*, whose missense mutations, insertions, deletions and duplications give rise to MutYH-associated polyposis, the most common colorectal cancer and polyposis syndrome. *MutYH* recognizes the mismatch 8-oxo-G:A and, through its action, restores the pair 8-oxo-G:C, that can be acted upon by OGG1, another BER glycosylase. The OGG1 polymorphism Ser326Cys is associated with the risk of lung cancer and increased risk of colorectal cancer; these findings confirm the roles of MutYH and OGG1 as essential players in the maintenance of genome stability against oxidative damage. In addition, the polymorphism Asp148Glu of APE1 seems to be associated with hypersensitivity to ionizing radiation and cancer risk and it can affect the prognosis of ovarian, gastro-oesophageal and pancreatico-biliary cancers [62–65]. Finally, polymorphisms of XRCC1, such as Arg399Gln

and Arg194Trp, are related to the risk of skin, upper aerodigestive and lung cancers; moreover, it is important to know that these genetic polymorphisms might be associated with overall survival and response to platinum-based chemotherapy in lung cancer patients [62, 66].

Another DNA repair pathway whose alterations are strongly related to tumour development is the MMR pathway. The Lynch syndrome, a tumour predisposition syndrome characterized by colorectal and endometrial cancer and other extracolonic malignancies, is indeed caused by monoallelic germline mutations in mismatch repair genes, such as *MLH1*, *MSH2*, *MSH6* and *PMS2*, while biallelic mutations lead to a more severe scenario called constitutional mismatch repair deficiency (CMMRD). Childhood onset of leukemia/lymphoma, brain tumours and other rare malignancies are all typical features of the CMMRD disease [67, 68].

Interestingly, distinct phenotypes and clinical manifestations are due to different *MMR* gene deficiencies; for example, an increased risk of colon cancer is particularly associated with *MLH1* mutations, whereas *MSH2* mutations have a higher incidence of extracolonic tumours. Also, as shown for clinical features, the chemotherapy resistance has different outcomes based on *MMR* gene loss; for example, the MSH2-deficient cells, but not MLH1-deficient, are sensitive to psoralen, a chemotherapic agent that induces DNA interstrand crosslinks [69].

These examples of DDR defective-associated cancers justify the strong interest in figuring out all the features related with the regulation of DDR to reach a deeper knowledge of cancer development and chemoresponse.

Targeting DNA Repair Enzymes in Cancer Therapy: The Concept of Synthetic Lethality

Somatic and hereditary mutations in DNA damage response (DDR) genes are thus associated with an increased cancer risk, but they can also offer new venues for cancer treatment. For example, the mutations of *BRCA1* and *BRCA2* genes, involved in double strand breaks repair via HR, are among the most studied, due to their strong correlation with breast and ovarian cancer development. Indeed, it was discovered that, due to their intrinsic deficiency in HR, these BRCA-deficient tumours are particularly sensitive to inhibitors of another DNA repair enzyme: poly(ADP-ribose) polymerase1 (PARP1).

PARP1 has the ability to bind single strand-breaks (SSBs) and facilitate their repair. Loss of PARP1 activity is supposed to cause formation of DNA-SSBs which are subsequently converted to double strand breaks (DSBs). In BRCA-positive cells, these DSBs are repaired by HR, but in BRCA1-or BRCA2- deficient cells they are presumed to accumulate leading to subsequent cells death [70, 71]. This discovery allowed the development of specific PARP1 inhibitors that, in the context of a BRCA1 or BRCA2 deficient genetic backgrounds, proved to be very effective in suppressing tumour growth in Phase I/II clinical trials. Such a synthetic

lethality approach, however, did not entirely meet the expectations and failed in Phase III clinical studies. An explanation for this behaviour could be the activation of an alternative DSB repair pathway, such as NHEJ, that allows the survival of HR-deficient cells. However, Patel et al. [71] turn this hypothesis upside down, demonstrating that disabling NHEJ diminished the genomic instability and lethality of PARP inhibition in HR-deficient cells rather than exacerbating it. In this context, an emerging role could be assumed by the X-family DNA polymerases; in fact, they are involved in many of the alternative pathways of the DSBs repair like the Microhomology-Mediated End Joining (MMEJ), Single-strand annealing (SSA), Break Induced Replication (BIR) and others. Recent results make polλ an ideal candidate for a new target therapy due to its ability to promote strand annealing and subsequent elongation between two DNA strands with limited homology (5–10 nt) [72].

New Candidates for Synthetic Lethality: Repair DNA Polymerases

The case of PARP1 inhibitors highlights a fundamental problem: namely, achieving a true synthetic lethality by suppressing a particular molecular function in the context of the high level of redundancy existing among the cellular metabolic pathways. A possibility to attain such a difficult goal might be to target enzymes common to different DNA repair pathways. The most obvious candidates are, of course, the DNA polymerases (pols). In fact, since DNA pols are essential to several repair pathways, their inhibition might potentially achieve a high level of tumour cell sensitization to chemotherapeutics.

Specialized DNA pols are required to bypass DNA damage lesions that would otherwise cause replication arrest and cell death. In recent years, a number of specialized DNA pols of the X and Y families have been identified. These are characterized by their ability to bypass different classes of lesions and to maintain a high degree of genetic fidelity by incorporating the nucleotide that would normally pair with the undamaged version of the base. Thus, under normal circumstances, specialized pols can be considered as agents that promote genomic stability. Trans-lesion synthesis (TLS), however, needs to be a highly regulated process because, when copying non-cognate lesions or undamaged DNA, the specialized pols have been shown to exhibit reduced fidelity. In addition to a role in mutagenesis, over-expression or increased activity of specialized pols could also result in enhanced TLS capability, allowing cancer cells to better cope with the high environmental stress that results from increased replication rates and higher level of oxidative damage. Moreover, increased TLS could provide cancer cells with an advantage in coping with the DNA damage resulting from the chemotherapeutic assault [73].

The Y-family members, in human cells, are DNA pol ι, DNA pol κ, DNA pol η and Rev1, each of them has a preference for catalysing DNA synthesis across certain kind of lesions. For example, the loss of DNA pol η reduces the efficiency to copy cis-syn cyclobutane dimer, one of the main lesion generated by sun exposure, and gives rise to the variant form of xeroderma pigmentosum (XP-V), a disease characterized by high susceptibility to sunlight-induced skin cancer. In addition, it has been shown that pol η has a key role in the cellular response to cisplatin and in the cellular resistance to this antitumour drug [74]. Another example is the role of pol ι in the induction of lung tumours, in fact pol ι knockout mice treated with urethane, a pulmonary adenoma inducer, did not develop cancer compared to wild type controls. Thus, it suggested that pol ι deficiency could lead to reduction of lung tumour [75]. On the other hand, Ziv et al. [76] discovered an important role of another Y-family enzyme, pol κ, in protecting cells from UV damages. Based on these findings, it is becoming clear that the mis-regulation of the Y-family members promotes genetic disorders and can be associated with a malignant phenotype [77].

Beside TLS, the BER and NHEJ DNA repair pathways seem to play a prominent role in promoting genetic instability in cancer cells. Central to these pathways are the X-family enzymes DNA pol β, λ, μ and TdT. TdT has the peculiar characteristic of elongating a single stranded DNA sequence without the need of a template strand. It normally acts during immunoglobulin and T cell receptor gene rearrangements, thus increasing the diversity of these molecules. A DNA pol with closely related amino acid sequence and functional domain organization to TdT is pol μ, whose roles in V(D)J recombination and NHEJ are well described. The over-expression of TdT and pol μ has been observed in several acute leukemia cells and in Non-Hodgkin's lymphomas, respectively, suggesting a possible role in tumourigenesis [78, 79].

Pol β is the smallest pol and is composed of a single 39 kDa polypeptide containing 335 amino acid residues. It is involved in BER pathways (short and long patch BER) and implicated in meiotic events associated with synapsis and recombination and SSB repair [80]. Mice carrying a target disruption of the pol gene show growth retardation and high perinatal lethality; histological examination of the embryos revealed defective neurogenesis, indicating that pol plays an essential role in neural development [81].

The over-expression of pol β has been found at both the mRNA and protein levels in many tumour types, in particular in uterus, ovary, prostate and stomach samples. In addition, its ectopic over-expression in cancer cells can increase mutagenesis and enhance resistance to chemotherapeutic agents, including cisplatin, while cancer cells deficient in pol are hypersensitive to oxaliplatin chemotherapy, indicating that BER impairment could affect the therapy outcome [73, 82]. Cells deficient in pol β converted into another kind of critical pro-apoptotic lesion during replication. These critical secondary DNA lesions are likely to be unrepaired DNA double strand breaks, which trigger apoptosis in a replication-dependent way by activating the mitochondrial death pathway, i.e. the decline of Bcl-2 level and activation of caspase-9 and caspase-3 [83].

Pol λ is a protein of 67–70 kDa which is expressed at highest level in the testis, ovary and fetal liver and it seems to be implicated in short patch BER repair, NHEJ, TLS over 8-oxo-G and V(D)J recombination [84, 85]. Despite the strong expression of pol λ seen in testis, POLL null mice were fertile and the only effect seemed to be a modification of heavy chain junctions during V(D)J recombination [86, 87]. Pol λ was found to be over-expressed at a significant extent in a range of different tumour types, albeit less frequently than pol β [73]. Recently, a new allelic variant of human pol λ has been described. It is the result of the amino acid change Arg 438 to Trp and it seems to have a reduced base substitution fidelity. Thus, the ectopic expression of R438W hPolλ variant in mammalian cells increases the mutation frequency, affects the DSB repair NHEJ pathway and generates chromosomal aberrations [88]. Another recent study reports that the pol λ protein level can be modulated in tumour cells, and in fact, NSCLC that express less protein amount are in a significantly more advanced stage [89]. Furthermore, it has been observed that more than 90 % of leukemic cells of acute lymphocytic leukemia and approximately 30 % of leukemic cells in chronic myelogenous leukemia crisis exhibit elevated TdT activity, which is associated with poor prognosis and response to chemotherapy and reduced survival time. Since leukemic cells also often over-express pol λ, which has been shown to possess a strong bona fide TdT-like activity, it is possible that both pol λ and TdT have an important role in tumourigenesis and progression of the acute leukemia [90]. In a recent study, pol λ seems to be involved in the incorporation of therapeutic nucleoside analogs into DNA during BER and NHEJ, thus it may have an impact on the cellular sensitivity to these compounds following DNA damage [91].

Recently, another DNA pol, member of the A-family, called DNA polymerase θ might be implicated in cancer development. It seems to be involved in tolerance of bulky adducts or in some DNA repair pathways such as BER, DNA interstrand crosslink and DNA break repair. Its overexpression is found in breast, colon and lung cancers and it is usually related to poor prognosis [92, 93]. In addition, a recent study demonstrated that DNA pol θ knockdown on a panel of tumour cell lines from different primary sites resulted in radiosensitization, whereas having little or no effects on normal tissue cell lines [94].

Development of Selective Inhibitors of Specialized DNA Polymerases

Currently, several classes of DNA pol inhibitors have been developed. Most of them are non-nucleosidic compounds of natural origin (polypeptides, fatty acid, triterpenoids, sulfolipids, polar lipids, secondary bile acids, phenalenone-derivates, anacardic acids, harbinatic acid, flavonoid derivates and pamoic acid), but only a few are enough specific and active to be potentially considered as drug candidates. Nucleoside analogs (NAs), on the other hand, mimicking the structure of the natural

nucleotides, can interact with the catalytic site of the pols and inhibit the DNA synthesis and/or repair by competing with the natural substrates. In addition, the lack of the 3′ hydroxyl group, typical feature of most NAs, prevents the elongation step, determining an abortive DNA replication and single strand breaks formation. Currently, eight NAs have been already approved by FDA for cancer treatment: mercaptopurine, thioguanine, fludarabine and cladribine (purines), cytarabine and gemcitabine (pyrimidines), fluorouracil and capecitabine (fluoropyrimidines); whereas clofarabine (CAFdA), nelarabine, immucillin H (BCX-1777, forodesine) and 8-chloroadenosine (8-Cl-Ado), all novel purine analogs are in advanced clinical phase. Unfortunately, NAs suffer from a number of drawbacks. First, they need to be subjected to three independent cellular phosphorylation steps to be converted to their active forms. Moreover, they can be easily degraded by other enzymes such as nucleosidases and phosphorylases and, finally, their inhibitory effects can affect also replicative polymerases, thus becoming toxic to normal cells [95, 96]. In this scenario, a novel diketo hexenoic acid (DKHA) analog was described as a selective non-nucleoside inhibitor of the template-independent activity of pol λ and TdT. Locatelli et al. [90] proved that this compound can selectively suppress cell proliferation of TdT+, but not Tdt^{-}, leukemic cells, holding the potential to be further developed as a novel antitumour agent.

Tying the Ends Together: Targeting Proliferation and Repair in Cancer Cells

The inhibition of different targets in order to obtain synthetic lethality within cancer cells could greatly ameliorate the efficacy of target therapy. In this way, it is possible to hypothesize the concomitant inhibition of targets that belong to different cellular process. A fascinating idea regards the targeting of factors that link signal transduction and DNA repair. In fact, oncogenic kinases activity is linked to DNA repair [97]. For example, it is known that tumours dependent on fusion kinases, like Bcr-Abl, present an elevated number of DNA double-strand breaks (DSBs). These are caused by high ROS levels generated by the altered cell metabolism and also by chemotherapy [98]. The DSBs repair processes require the activity of Werner Helicase/Exonuclease (WRN) which plays a critical role in optimizing DSB repair mechanisms due to its DNA end-processing activities. Slupianek et al. [99] showed that Bcr-Abl is able to enhance WRN expression via c-Myc–induced transactivation and Bcl-xL–dependent inhibition of caspase-mediated cleavage. Moreover, the Bcr-Abl kinase forms a complex with WRN protein which results in constitutive phosphorylation and activation of WRN itself. As result, WRN promotes the survival of Bcr-Abl positive leukemia cells under oxidative and genotoxic stress. Furthermore, in these cells, WRN promote alternative DSBs repair mechanisms such as Homologous Recombination (HR) and Single Strand Annealing (SSA). Additionally, in Bcr-Abl positive leukemia cells,

WRN caused aberrant Non Homologous End Joining (NHEJ) repair products [100]. Altogether, these effects can promote survival of cancer cells, inducing at the same time the accumulation of genetic aberrations in CML, a mechanism by which cancer cells could acquire resistance mutation to the therapy.

Other studies underline a strict interplay between signal transduction proteins and DNA repair factors. For example, inhibition of Chk1, a STK that is activated in response to DNA damage, was found to be synthetically lethal with Src or ERK inhibitors in myeloma and leukemia cells, respectively [101–103]. Also, EGFR was found to interact with BRCA1 in highly aggressive breast cancers. Concomitant inhibition of EGFR with Lapatinib and PARP1 with ABT-888 led to transient DSBs repair deficiency which resulted in the activation of the intrinsic pathway of apoptosis [104].

Casein Kinase II (CK2) is an ubiquitously expressed STK, whose activity is implicated in cell growth and proliferation. Its overexpression is linked to cancerogenesis and attenuated apoptosis induced by chemotherapeutic drugs [105], posing CK2 as an attractive target for cancer treatment. In particular, CK2 inhibits the tumour suppressor activity of promyelocytic leukemia protein (PML) and phosphatase and tensin homology protein (PTEN) [106]. CK2 phosphorylates

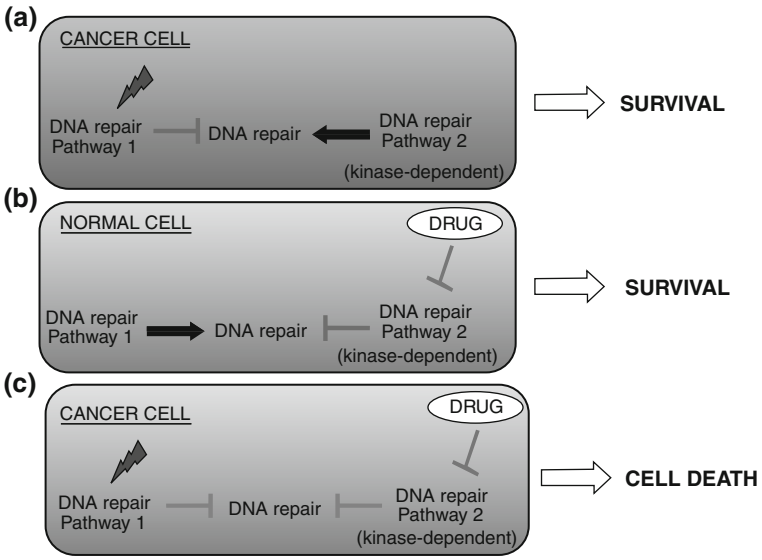

Fig. 1 *The synthetic lethality approach through targeting DNA repair and signal transduction*. **a** In a cancer cell, a defect in a DNA repair pathway can be overcome through the induction of an alternative pathway, often under the control of signal-transducing kinases. **b** In a normal cell, where all the DNA repair pathways are effective, inhibition of one of them, either by targeting the apical kinase or directly the repair DNA polymerase, will not affect its survival. **c** In a cancer cell, already defective for a given DNA repair mechanism, inhibition of the existing alternative pathways, either by targeting the apical kinase or directly the repair DNA polymerase, will result in apoptosis

XRCC1, a scaffold protein that plays a critical role in DNA base excision repair (BER) by interacting with Ligase III (LigIII). Phosphorylation of XRCC1 in human cell extracts is required for XRCC1-Lig III complex stability and phosphorylation reduction leads to DSBs accumulation [107]. Thus, inhibition of CK2 would lead not only to apoptosis in response to chemotherapeutic drugs, but also in accumulation of DSBs that will enhance the apoptotic effects of the therapy.

Conclusions

The above-discussed examples highlight the intimate connections between the proliferative signal transduction pathways and the DNA repair. In such a context, inhibition of specialized DNA pols, that act in different repair mechanisms, will reduce the ability of cancer cells to cope with the genotoxic stress imposed by proliferation and/or cancer treatment. Thus, simultaneously targeting signal transduction and DNA repair pathways will drive very efficiently cancer cells to apoptosis (Fig. 1). The investigation of the interplay between signal transduction pathways and DNA repair pathways in cancer cells can lead to the identification of novel therapeutical targets and to the design of cancer treatment strategies that avoid the onset of resistance to the therapy.

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Conflicts of Interest No potential conflicts of interest were disclosed.

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