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Screening of Natural Products for Antimicrobial Agents

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Antimicrobial research is geared toward the discovery and development of novel chemical structures such as therapeutic antimicrobial agents. The continuing problem of development of resistance to existing antibacterial agents and the dearth of good antifungal agents motivates this effort toward innovation. Selection of possible new enzyme targets for antibiotic inhibition may be made on theoretical grounds, but it appears premature to select any single, previously unvalidated target for the intensive study required for rational drug design. Instead, a broad screen of chemical entities can be undertaken, dedicated to the discovery of novel antimicrobial inhibitors. A number of target areas are under investigation, including fungal mRNA splicing and bacterial DNA synthesis. A major part of the endeavor is in the historically productive area of natural product screening. To make the best use of natural product resources for the discovery of novel antibiotics, a balance is struck between screening for inhibitors of rationally chosen targets for which clinically useful inhibitors are not yet available, and screening more broadly to ensure that rare activities of unanticipated mode-of-action are not missed.

The Need for Innovation

Antimicrobial research is geared toward the discovery and development of novel antibacterial and antifungal agents. In parallel, efforts continue in the development of next-generation chemotherapeutic agents through chemical design. A major goal, however, is that of finding new chemical entities rather than continuing the search for new members of already defined chemical classes. Thus, a primary aim is innovation, the discovery and development of new classes of chemical structures as therapeutic antimicrobial agents.

In bacterial disease, where potent, relatively safe chemotherapeutic alternatives exist, the need continues for broad spectrum antibiotics not subject to the multiplicity of resistance mechanisms which have developed among pathogens. Furthermore, there remains a need for orally administered, broad spectrum, non-allergenic antibiotics appropriate for pediatric use, as well as novel, safe, bactericidal broad spectrum parenterally administered agents. Problem pathogens continue to arise which are multiply drug resistant and which must be the targets of aggressive empirical therapy. In fungal disease there are no non-toxic fungicidal drugs on the market, and needs for a broad spectrum, safe, preferably orally administered, fungicidal drug are growing due to the emergence of super-infecting organisms in immuno-compromised, debilitated or elderly patients and the prophylactic/therapeutic challenges caused by AIDS.

The realities for the pharmaceutical industry are such that the cost of development of any new drug requires a high probability of commercial viability, long patent life and a good competitive profile – all of which are characteristics of breakthrough novel entities.

Rational Drug Design

In the search for such breakthrough agents in this age of "rational drug design" we might select a target enzyme on theoretical grounds, study its three dimensional crystal structure, its interactions with small molecules, and design novel inhibitors for it – as attempted in other therapeutic areas. In general, antimicrobial chemotherapy has not reached this stage, not for lack of technology, but rather due to the complexity of the question. In other therapeutic areas, inhibition of a given enzyme can be shown to be directly responsible for alleviation

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of a disease state, for example inhibitors of angiotensin converting enzyme lower blood pressure, inhibitors of hydroxymethylglutaryl-coenzyme A reductase lower serum cholesterol. This is not true for antimicrobial agents. Eradication of the organism is neccessary for alleviating infection. As discussed below, it is not straightforward to select an enzyme whose inhibition leads inexorably to the death of the pathogen.

The rational design of antibacterial agents has been approached using known enzyme inhibitors, substrates or transition state intermediates as models, leading to design of inhibitors of enzymes such as D-alanyl-D-alanine ligase (1), dihydrofolate reductase (2) and CMP-KDO synthetase (3, 4). In many instances, potent enzyme inhibitors were successfully designed and synthesized. Their antibacterial activity howver is modest. One explanation for the lack of potent antibacterial activity is limited uptake of inhibitor by cells, an issue too complex to be addressed here, but of great importance to the eventual design of antimicrobial drugs, representing, in a sense, a separate (and manifold) target.

Screening for inhibitors of rationally chosen microbial targets if thus favored over rational design of new chemical classes of antibiotics.

The Nature of Antimicrobial Targets

In the history of discovery and development of antibiotics, most structural classes were found before their targets were known, and in fact many became the instruments whereby corresponding macromolecular metabolic pathways of synthesis were elucidated. For example, the nature and biosynthesis of the bacterial cell wall was defined largely through studies of the effects of β–lactam antibiotics on its synthesis and assembly (5). Similarly, many of the functional steps of protein synthesis have been dissected through the use of antibiotics – even though the nature of the catalytic activities involved and their residence on protein, RNA or both remain unclear (6).

Analysis of the modes of action of clinically successful classes of antibiotics indicates that, in general, effective antibiotics are those which interfere with steps in the synthesis of cellular macromolecules. The synthetic machinery and substrates for these processes are often complex. Of the known bactericidal antibiotic classes, it appears that few are good single-entity therapeutic drugs by virtue of their interaction with single catalytic sites. Rather, they exert their effect in a number of more complicated ways. They may bind tightly to a substrate, as does vancomycin (7); interact with a complex of enzyme and DNA substrate, as do the quinolones (8); influence several steps in the synthetic process, as do the aminoglycosides (9); or inhibit multiple catalytic sites, as do the β-lactam antibiotics. The last example is strikingly illustrated by a study of the role of the penicillin-binding proteins (PBPs) of *Staphylococcus aureus* in the induction of bacteriolysis by β–lactam antibiotics (10). At least two staphylococcal PBPs must be blocked in order to induce a bacteriolytic effect: PBP1 must be blocked together with PBP2, PBP3 or both.

The complex nature of the targets of many existing antibiotics indicates that a priori selection of a single enzyme-target for drug design or discovery may be difficult. Moreover, single enzymes can be poor targets for antibiotic monotherapy due to resistance arising by alterations of the target site which prevent antibiotic recognition. An example of this is the failure of monotherapy with rifampicin, an inhibitor of bacterial DNA-dependent RNA polymerase, due to selection during treatment of organisms with mutant RNA polymerase (11). Rifampicin can be used successfully, however, in combination with other antibiotics (12). Rational combinations may be a key to development of new single-enzyme inhibitors.

The choice of novel primary targets for antibiotic intervention is further complicated by the observation that cell death, as opposed to cessation of growth, does not appear to be a necessary consequence of the interaction of inhibitor and target. Studies with bactericidal agents such as β-lactams (13, 14), aminoglycosides (9), and quinolones (15, 16) indicate that the primary interaction, although necessary, is not sufficient to cause loss of viability. Instead, it may trigger off a cascade of events or a lethal response by the organism. Since there is as yet an incomplete understanding of the nature of such lethal mechanisms, it may be difficult to predict which primary targets to pursue, although a genetic approach may be productive.

Rational Targets for Antibiotic Inhibition

The foregoing discussion argues that it is difficult to select any single enzyme as a novel target for antibiotic intervention based on studies of existing classes of antibiotics. However, both classical and molecular genetics as well as biochemistry can be utilized to help project which pathways of microbial metabolism might be suitable new targets for chemotherapeutic intervention. The abundance of conditional

lethal mutations in bacteria and fungi reveals a variety of sites in macromolecular synthesis which might be target candidates. In many cases, temperature sensitive alleles define enzymes which are essential to cell viability. Using modern molecular genetic techniques, it is feasible to create dominant lethal mutations defining a set of enzymes which, when altered, are capable of actively interfering with normal cellular metabolism in a way that triggers death. Biochemical and molecular genetic studies can be pursued to determine whether a putative target is different from or lacks a mammalian counterpart.

Table 1 summarizes those pathways of macromolecular metabolism which are proven targets for antibiotic intervention and others worthy of investigation. Among the existing antibacterial agents, it is clear that protein and cell wall biosynthesis are the targets of the widest variety of natural product classes. It is likely, however, that there are steps in many other pathways which, if inhibited, would lead to cell death. Upon rational selection of target enzymes and pathways, efforts to find new agents can be initiated. Two such possible target areas are RNA-splicing in fungi and DNA replication in bacteria.

Candidate Target Pathways

Messenger RNA splicing in fungi is an attractive target for the development of novel antifungal antibiotics. No inhibitors of this pathway are known, thus any compound selectively inhibiting any step in the pathway has a high probability of novelty. Genetic studies have shown that mRNA splicing is essential in *Saccharomyces cerevisiae* (17). There are differences between the pathway in yeast and its mammalian counterpart. *Saccharomyces cerevisiae* does not splice higher eukaryotic introns and the RNA sequences required for splicing differ between the two systems (18). Furthermore, the systems differ in size and abundance of small nuclear RNAs and in the size of spliceosome complexes (19, 20). While *Saccharomyces cerevisiae* is not a pathogen, it has been shown that the β-tubulin gene of *Candida albicans*, an important fungal pathogen, contains an intron with yeast splicing-consensus sequences and is spliced in *Saccharomyces cerevisiae*. Thus, the splicing pathway meets many criteria for target candidacy.

In the area of antibacterial antibiotic discovery, the DNA replication pathway is a likely target. More than twenty years of physiological, genetic and biochemical study has led to a very detailed understanding of DNA replication in *Escherichia coli*. The process is better understood in this organism than in any other (21, 22). A number of enzymatic steps in bacterial DNA replication, listed in Table 2, may be considered as possible antibiotic targets. Conditional lethal mutations in many of these enzymes indicate their essentiality and the likelihood that their inhibition will be lethal. Mammalian counterparts for the DnaA, DnaB, DnaC and primase proteins are either clearly different or not known to exist, thus making them good can-

Table 1: Pathways of macromolecular metabolism as antimicrobial targets.

Pathway/function	Antibacterial antibiotics	Antifungal antibiotics
Protein synthesis	tetracycline, streptomycin, fusidic acid, chloramphenicol, erythromycin, kirromycin, lincomycin, oxazoladinone	–
Cell wall synthesis	β-lactams, glycopeptides, fosfomycin, cycloserine, moenomycin, bacitracin, daptomycin	echinocandin, nikkomycin
Membrane integrity	polymyxin, gramicidin	nystatin, amphotericin
RNA synthesis	rifamycin, streptolydigin, sorangicins	5-fluoro-cytosine
DNA synthesis	novobiocin, quinolones, trimethoprim/sulfamethoxazole	–
Microtubule function	–	griseofulvin
Lipid/membrane synthesis	amino-dideoxy KDO	–
Cell division	–	–
Vitamin/ion uptake	–	–
Protein secretion	–	–
RNA processing	–	–
DNA methylation	–	–

Table 2: Possible antimicrobial enzyme targets in the DNA replication pathway: *Escherichia coli* enzymes and mammalian counterparts.

Escherichia coli enzyme	Function	Mammalian counterpart
DnaA	ori-binding	
	ori-opening	SV 40 T-antigen
	helicase guide	or?
DnaB +	helicase	
DnaC	primase guide	no analog
SSB	ssDNA binding	CF1
Gyrase	topoisomerase	Topo I or Topo II
Primase	priming	Pol α
Pol III + β subunit	polymerase (lagging strand)	
	processivity	CF-3
	polymerase (leading strand)	Pol δ
	processivity	PCNA
Pol I & RNase H	primer removal	RNase H &?
Ligase	chain joining	Ligase
Ter-binding protein	termination factor	?

didates for inhibitors displaying differential toxicity. While DNA gyrase and topoisomerase II have similar functions, inhibitors of bacterial gyrase (the quinolone antibiotics) and mammalian topoisomerase II (epipodophyllotoxins, adriamycin, etc) have been shown to be highly selective inhibitors of the respective enzymes. To take full advantage of this target pathway all of the replication proteins of *Escherichia coli* can be purified and used to develop in vitro DNA replication systems. These biochemical assays may then be used to elucidate the specific mode of action of compounds identified. The biochemical approach can also aid as well in target assessment, screen design and eventually structure-activity studies.

The Nature of Natural Products

In order to find new antimicrobial agents and validate predictions of theoretically good targets, a variety of entities both from chemical collections and natural products are screened for leads. Natural products have been a rich source of medicinally active compounds, including most of the clinically useful antimicrobial agents (Table 3). It is impressive that in most of these classes, the actual natural products themselves were effective, had low enough toxicity, a broad enough spectrum and sufficiently good pharmacokinetics to be clinically useful without chemical modification. It should be noted, however, that many thousands more have been described which are not very potent or are toxic to the host.

What is the nature of natural products, the so-called "secondary metabolites" of bacteria, fungi, and plants? Over the years, potent antimicrobial agents have been found, but the incidence of new structural classes appears to have declined, as indicated in Table 3. This may be due to the fact that discoveries of the 1970s and 1980s have not yet been recognized for their utility, or that standards have changed, precluding development of some unlisted classes. Alternatively, this could be due to imperfect screening methodology or to exhaustion of the source. We need to know how to select new entities, avoiding the very large number already described.

Recent reviews (23, 24) have summarized many hypotheses for the function in producing organisms of secondary metabolites, a subject of controversy over the entire age of antibiotic research. In the last decade, opinions and theories of workers in the field have varied widely. Representing one end of the spectrum, Zähner et al. (25) theorize that secondary metabolites provide a sort of "elbow room in biochemical evolution" where novel, even infinite combinations of reactions may occur to produce an unlimited number of [different] secondary metabolites which, as long as they are not disadvantageous to the organism, can be conserved for some [amount of evolutionary] time and perhaps eliminated. Those conferring some advantage to the organism in its intermediary metabolism would be protected from elimination and hence selected for. Zähner et al. (25) concluded that we should "... rid ourselves of the simplistic idea that antibiotics are formed as defence mechanisms, and recognize instead

Table 3: Structural classes of natural product antimicrobial agents, useful drugs of these classes and dates of disclosure.

Class	Year	Useful drug	Year
Peptides	1939	polymyxin	1939
		bacitracin	1952
Griseofulvin	1939	griseofulvin	1939
Penicillins	1940	penicillin G	1940
Steroids	1943	fusidic acid	1962
Aminoglycosides	1944	streptomycin	1944
Chloramphenicols	1947	chloramphenicol	1947
Tetracyclines	1948	chlortetracycline	1948
Macrolides	1950	erythromycin	1952
Polyenes	1950	nystatin	1950
		amphotericin	1955
Glycopeptides	1955	vancomycin	1955
Lincosamides	1955	lincomycin	1962
Cycloserine	1955	cycloserine	1955
Streptolydigin	1955	streptolydigin	1955
Coumarins	1956	novobiocin	1955
Rifamycins	1959	rifamycin SV[a]	1961
Cephalosporins	1962	cephalothin[a]	1962
Glycolipids	1965	moenomycin[b]	1965
Polyoxins	1965	nikkomycins[b]	1965
Phosphonates	1969	fosfomycin	1972
Elfamycins	1972	kirromycin[b]	1972
Cephamycins	1973	cefoxitin[a]	1971
Monobactams	1973	aztreonam[c]	1981
Carbapenems	1976	thienamycin	1979
Lipopeptides	1980	echinocandin B	1974
		daptomycin	1980

[a]Semi-synthetic.
[b]Veterinary or agricultural use.
[c]Totally synthetic.

that antibiotics are nothing more than secondary metabolites which possess, more or less incidentally, an antibiotic effect ..."

At the other end of the spectrum, Williams et al. (26), defining a secondary metabolite as "a substance appearing to have no explicit role in the internal economy of the organism that produces it", espouse one of the hypotheses summarized by Haslam (23) that, "[natural products] serve the producing organisms by improving their survival fitness ... The ability to synthesize an array of secondary metabolites which may repel or attract other organisms has *evolved* as one facet of the organism's strategy for survival". Williams et al. (26) present many good examples to bolster their argument that secondary metabolites are the products of evolution, being selected for their ability to inhibit organisms outside the producer. But they go further in rejecting all other possible theories of secondary metabolism and "roles"for such metabolites. They reject the broader concept that secondary metabolites have evolved under the influences of the producer's environment, either internal or external, due to the constraining definition that the substance must appear "to have no explicit role internal to the cell".

There is, indeed, a growing body of work, reviewed recently by Chater (27), showing that in each of the 23 cases where genes for antibiotic production by streptomycetes have been cloned, the synthetic genes are clustered and resistance genes are generally linked to these clusters. Such elaborate organisation argues strongly for evolution and selection of these antibiotics. It can be asked if this is true of all secondary metabolites.

It seems most reasonable to adopt the broad concept that natural products have been subject to evolutionary selection, both internal and external to the organism, admitting that in many cases their purposes have not yet been discovered. Clearly, too, the enzymes of secondary metabolism have some flexibility in substrate recognition, allowing a degree of variation of which we may take advantage. While the pool of natural products may be non-random, it is certainly large. By expanding the diversity of the producing organisms and the ways in which they are fermented, new compounds with the characteristics we desire will hopefully be found.

Screening Strategies: An Overview

The nature of natural products (or secondary metabolites), which we are in the process of exploring, must inform our choice of strategies for screening. To best use natural product resources, efforts to discover novel agents should be two-pronged, covering both the theoretical as well as empirical approaches. To find novel structures, screens can be devised for inhibitors of enzymes and pathways selected on the basis of essentiality for the organism and the likelihood that inhibitors of such targets will be non-toxic to the host. More empirically, screening strategies can be used that are geared toward the recognition of rare but novel structures that are potent and "fully-evolved".

In the selective screening approach, theoretical targets selected as candidates based on physiology and genetics can be screened with great sensitivity and specificity. While there may be no "fully-evolved" antibiotics in nature which inhibit the chosen targets, "imperfect" compounds (low potency, poor permeability, solubility, stability), or those present in very low concentration can be found and used to validate targets and serve as leads for improvement by synthetic chemists. Not only medicinal chemistry, but fermentation development, biotransformation and genetic manipulation of the producing organism can yield improvements in

the properties of a lead. To be successful, this highly selective approach should be utilized in the longer term goal of understanding and selecting molecular targets, by committing itself to the isolation, purification and characterization of these non-ideal leads.

This approach is very attractive for the discovery of new antifungal agents, since the likelihood of toxicity of therapeutic agents directed against these eukaryotic pathogens is greater than with antibacterial agents. Knowledge of the fungal target and its mammalian counterpart is extremely useful in supporting the efforts of medicinal chemistry in dissecting mechanism based toxicity away from general toxicity.

Natural products can also be screened empirically for novel structures and/or activities, previously undetected "fully-evolved" antimicrobial agents hopefully being discovered. Given the key, pathways and enzymes not previously recognized become effective new rational targets for antibiotic intervention. The antibiotic-target interactions of these new agents can also be characterized to the extent that they become useful models for the rational design of next-generation inhibitors. This is essentially a continuation of the classic mode of antibacterial discovery, taking advantage of modern methodology for improved assay design and selection of producing organisms. Critical issues in this type of approach are the difficulties of dealing with the large number of more commonly seen, previously described chemical classes ("dereplication") and the possibility of toxicity which, without a clearcut understanding of the mechanism of action of a compound, will hamper its further development. Broad based screening methods can take a variety of forms – generally multi-layered, including primary screening for antimicrobial activity against resistant pathogens or general inhibition of entire pathways of macromolecular synthesis. Commonly seen activities and toxic compounds can be eliminated by various counterscreens and classification techniques, and then further characterized to define antimicrobial spectrum and determine the mode of action. Improved technology should also allow the future development of multi-dimensional classification arrays which fingerprint compounds by biological and physical properties, using computer-aided systems for analysis of novelty.

In practice, these two approaches may overlap. Both require a robust classification scheme to deal with screen output. Both also require strong biochemical support to dissect and clarify target-inhibitor interactions and to provide biochemical and molecular insight into efforts to improve leads using medicinal chemistry.

In actuality, present screening strategies have derived historically from attempts to narrow the number of "actives" needing to be dealt with and classified, when screening empirically. By establishing general screens directed at, say, inhibitors of cell wall biosynthesis, and designing classification techniques to readily differentiate the common types of compounds selected (β–lactams, fosfomycin, moenomycin, glycopeptides, etc), the uncommon outliers were spotlighted for further study, structure determination and elucidation of their precise mode of action. With a more sophisticated understanding of the biochemistry of cell wall biosynthesis which resulted from the study of these new agents, selection of single enzymes as screening targets became feasible. Thus, mode-of-action screening, first devised as a means of classification, evolved into a quest for exquisite selectivity. Screening for inhibitors of new targets chosen on theoretical grounds is an extension of this highly selective approach. However, reliance on a few highly selective targets may compromise the usefulness of a broad based natural product screening effort. To make the best use of these resources for the discovery of novel antibiotics, a balance must be struck between screening for inhibitors of rationally chosen specific targets for which clinically useful inhibitors are not yet available, and screening more broadly to ensure that the rare activities of unanticipated mode of action are not missed.

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