УДК 616—093/098:577.15

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PURINE SALVAGE ENZYMES AS TARGETS FOR THE CHEMOTHERAPEUTIC TREATMENT OF PARASITIC DISEASES

The review analyses the results showing that technological advances in studying the specific target molecules in a cell allow to develop new effective drugs for the treatment of a number of human maladies. The new approach to the drug design is based in the data of enzyme structure.

Rational drug discovery/desing. The traditional approach to the discovery of new drugs has involved the screening of large numbers of compounds or extracts for bioactivity against specific pathogens. This more or less random approach has usually been conducted without knowledge of the molecular targets within the pathogen or mode of action of the drugs. Host toxicity is usually determined empirically, only after a bioactive substance has been identified.

Another, more systematic approach to drug discovery begins with the identification of a molecular target within the pathogenic organism. Targets are usually selected because their functions are pivotal for survival and thus, inhibition of those functions may be lethal for the pathogenic organism. Appropriate targets may be either enzymes that catalyze unique metabolic pathways or enzymes that are present in both the host and the pathogen. In the latter case, the enzymes may be pivotal only for survival of the pathogen, or they might be of equal importance for both the host and pathogen but are suitable targets because of differences in the pharmacological responses to specific drugs. For all of these situations, after the target molecule has been selected, the objective is to identify details of molecular structure and/or function that can be exploited in the discovery or design of low molecular weight compounds that will selectively bind to the target molecule of the pathogen. Well-designed compounds should be highly selective in their affinity for the target molecule of the pathogen, as compared to molecules of the host and thus may be used at therapeutic levels that are non-toxic to the host.

Technological approaches to rational drug desing. If a target molecule is an enzyme, traditional biochemical procedures are appropriate for analyzing enzyme/drug interactions. For example, kinetic studies of the affects of various concentrations of a drug as an inhibitor of the initial velocity of an enzyme catalyzed reaction, provide information about whether a compound competes for binding to the active site of an enzyme or an enzyme substrate complex [1]. Such information can be useful for the design of novel inhibitors. Furthermore, steady state kinetic studies can enable the determination of the concentration for a compound (K_i) at which half of the enzyme should be bound to the compound. This concentration is directly related to the affinity of the compound for the target molecule and is the minimum concentration that might be expected to have any possibility of partially blocking the enzyme catalyzed reaction believed to be pivotal for the survival of the pathogenic organism. Although K_is can be determined using impure enzyme, this value, determined *in vitro*, can be more than an order of magnitude below that required *in*

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vivo to kill half of the pathogenic organisms [2]. However, K_is provide physical constants for quantitating the selective affinity of compounds for the target enzyme of a pathogen, as opposed to the homologous enzyme of a host organism. For enzymes that are unique to a pathogen, the K_i provides a minimum concentration for testing the *in vivo* effectiveness and pharmacological properties of a compound.

Enzyme structure based drug design utilizes structural details to design inhibitors selective for the enzyme of the pathogen. By definition, the determination of three-dimensional structure of the target enzyme is necessary. This can require the purification of milligram quantities of the target enzymes from both the host and the pathogen. Since many target molecules are in relatively low abundance within the cells of the host or pathogen, purification of these molecules from the native source is often impractical. However, developments in molecular biology have enabled the cloning of cDNA and the expression of high levels of enzymes within bacteria, yeast, or insect cells. For example, at present, it is possible to produce recombinant enzymes at 20—60 % of the total soluble proteins in bacteria [3—6]. Thus, the acquisition of quantities of protein adequate for structural analysis (i. e. via NMR or X-ray crystallography) is less problematical today than it has been in the recent past.

Selective inhibitors (often referred to as «lead compounds» or «leads») having an affinity for a target enzyme may already be known. However, even if «leads» have not formerly been identified, three-dimensional structure provides physical data that can be exploited, using computers and computational chemistry, in the selection of compounds that may bind to the active site of an enzyme, from among thousands of commercially available compounds [7]. Occassionally, the target enzyme may be similar enough to a related enzyme whose structure is known to enable the use of a model of the enzyme's structure rather than the actual 3-dimensional structure for the computer based selection of new leads [8]. Empirical testing (kinetic studies as described above) will demonstrate if any of these compounds are good «leads» with an affinity for the active site of the target enzyme. Also, co-crystallization of the lead compounds with the target enzymes of both the host and the pathogen enables three dimensional analyses and the determination of molecular contacts and atomic interactions between the lead compounds and the target enzymes. This type of analysis may reveal differences in the enzymes of the host and pathogen that can be exploited in the re-design of leads to enhance their affinity for the target enzyme of the pathogen [9—11]. Thus, iterative crystallography, lead re-design, and inhibitor testing both *in vitro* and *in vivo* can enable the design of a potent selective inhibitor of a target enzyme of a pathogen. In theory, inhibitors designed this way have a better chance for being developed as an effective and non toxic agent for the treatment of the disease caused by the pathogen.

Molecular targets of parasites for rationally designed drugs. The vast majority of drugs in use today, for the treatment of diseases caused by parasites, were identified by traditional methods requiring the random screening of large numbers of compounds for bioactivity against the parasites. Subsequently, the molecular targets of a few of these drugs have been determined. For example, dihydrofolate reductase (DHFR) has been shown to be the target of compounds used in the treatment of a number of diseases. Several compounds, such as chloroguanide, pyrimethamine, and trimethoprim, which bind to DHFR's are in therapeutic use for the treatment of diseases caused by parasites [12]. Furthermore, various other antifolates are being tested for the treatment of infections caused by *Pneumocystis carinii* [13—14] and *Toxoplasma gondii* [13, 15]. These two infectious agents are increasingly important because they often are problematical for immunocompromised patients suffering from AIDS or recovering from an organ transplant. Also, antifolates are used for the treatment of *Plasmodium falciparum*, an etiologic agent for malaria [12, 16]. In addition to DHFR, other molecular targets have been identified

for drugs used in the treatment of parasitic diseases. These include the ornithine decarboxylase of trypanosomes [17] and the heme polymerase of malarial parasites [18—19]. Rational approaches to drug discovery and design, as described above, can and are being employed to discover novel, potent, nootoxic compounds that will be more effective than existing approved drugs for the treatment of diseases caused by parasites.

Purine salvage enzymes as a target for rational drug discovery/desing. Due to their pivotal role for the survival of parasites, purine salvage enzymes were proposed more than 25 years ago as potential targets for the chemotherapeutic treatment of malaria [20, 21]. However, unlike DHFR, purine salvage enzymes have yet to be demonstrated to be the target of an approved drug used in the treatment of a disease caused by a pathogen. During the past two decades, the purine salvage enzymes of a number of different parasites have been investigated. These studies include enzymes from the etiologic agents for human leishmaniasis [22], giardiasis [23], Chagas' disease [24], and schistosomiasis [25], as well as bovine tritrichomoniasis [26]. These studies show that the parasites examined lack the anabolic pathways needed for the *de novo* synthesis of purines. Thus, these organisms are forced to rely exclusively upon salvage pathways for the purines [guanosine triphosphate (GTP) and adenosine triphosphate (ATP)] needed in RNA and DNA synthesis and for high energy phosphate bonds to drive cellular metabolism.

Hypoxanthine phosphoribosyltransferase [HPRT; IMP: pyrophosphate phosphoribosyltransferase, ES 2.4.2.8, also hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT)] is a purine salvage enzyme that has been studied extensively. Complementary DNA (cDNA) encoding the human, schistosomal, malarial, trypanosomal, tritrichomonal, and bacterial HPRT's have been cloned and sequenced ([27—32], respectively). The human HPRT has been a subject of extensive investigation because defects in this enzyme are known to be responsible for genetically inherited gout and Lesch—Nyhan syndrome in humans [33, 34]. The symptoms of these diseases, which range from deleterious to lethal, result from inactivation of the human enzyme. This emplasizes the importance of trying to minimize the interactions of drugs with the HPRT of mammalian hosts.

Leads for drugs targeted to HPRT's. The long range objective of the majority of the investigations of parasite purine salvage pathways is to discover or design compounds that will selectively inhibit the activity of a pivotal enzyme. The hope is that such a compound could be developed as a drug in the treatment of the parasitic disease. However, only in recent years has significant progress been made toward the identification of lead compounds that selectively target the purine salvage ensymes of any parasite [2, 22, 35].

Allopurinol is a relative non-toxic analog of xanthine that is approved for use in the treatment of gout in humans. In humans the target of allopurinol is the xanthine oxidase enzyme which converts xanthine to uric acid. Allopurinol has also been shown to kill leishmanial parasites [36]. The drug is salvaged by the PHRT enzyme into the nucleotide pools and is eventually incorporated into RNA which leads indirectly to an inhibition of protein synthesis. This mechanism is postulated to account for the antiparasitic action of allopurinol [36]. Thus, allopurinol is probably effective in the treatment of Leishmaniasis because it is salvaged and incorporated into RNA by the parasite but not by the host. These results show promise that other substrate analogs, that will selectively bind to the HPRT's of parasites, might be designed or discovered.

Queen et al. [2] demonstrated that 6-mercaptopurine (an analog of hypoxanthine) and 6-thioguanine were «potent competitive inhibitors» of the malarial HPRT. Six-mercaptopurine is metabolized to 6-thiouric acid (6-mercapto-2,8-purinediol) in humans [37]. Both 6-mercaptopurine and 6-thioguanine have been used therapeutically as antineoplastics in humans

[37]. Kinetic studies indicate that both of these compounds may be slightly selective in their inhibition of the malarial HPRT as opposed to the human enzyme. Thus, these compounds may be good leads for drugs targeted to the HPRT of parasites responsible for human malaria.

Recently, a new method has been reported for screening for leads targeted to the HPRT's of parasites [35]. This method is referred to as comparative complement selection and involves the rapid screening in bacteria of purine analogs for the inhibition of the recombinant HPRT's of parasites as compared to the recombinant human enzyme. The procedure uses the activity of a recombinant HPRT to complement genetic deficiencies of the host bacteria. The bacteria are unable to grow unless they are expressing a recombinant HPRT. Thus, the effects of compounds on the growth of these bacteria can be screened employing sterile, blank antibiotic testing discs and methods similar to those used in standard antibiotic susceptibility assays. Complement selection alone enables the identification of compounds that affect the growth of bacteria expressing a particular enzyme. However, direct comparisons with the effects on bacteria expressing the recombinant human enzyme enable the identification of compounds that selectively target the enzymes of the parasites.

Three-dimensional analysis: a prerequisite to enzyme structure based drug design. Since most HPRT's are functional as dimers, with molecular weights in excess of 40 kDa, the only practical method for determining three-dimensional structure requires crystallization followed by analyses of X-ray diffraction patterns. As of this date, the three dimensional structure for a purine salvage enzyme has yet to be reported in the literature, although the structure of orotate phosphoribosyltransferase, a distantly related enzyme, was recently reported [38]. However, several laboratories are actively working on the structure for a number of different HPRT's. For example, crystals which diffract X-ray to a resolution of less than 3 Å have been generated for both the human and schistosomal HPRT's (Focia' and Fletterick, personal communication). Thus, the structures of several HPRT's may be available in the not too distant future. As soon as these structures are available, will be possible to analyze the binding of «lead compounds» identified in the complement selection assay and to move directly into the next phase of enzyme structure based inhibitor design and refinement.

The development of resistance to new drugs. Microbial pathogens, including parasites, are notorious for their ability to develop resistance to drugs. It is for this reason that so much effort is being directed today toward the development of vaccines for the treatment of parasitic diseases. However, parasites seem to be extraordinarily adapted to surviving the immune responses of their hosts. This helps to explain why there is still not a single, completely effective vaccine in use today for the treatment of parasitic disease of humans.

The advantage of the rational approach to the discovery or design of new drugs, over the traditional random screening method, is that the target of the drug is known. If the target is an enzyme, it will need to be cloned and expressed to enable 3-dimensional and kinetic studies. Thus, even before a single new drug has been discovered, there is an normous side benefit that will enable the rapid development of second generation drugs to treat first generation drug-resistant strains that may appear in the future. Specifically, PCR primers can be designed that will enable the amplification and re-cloning of cDNA encoding the target enzyme of drug resistant strains using available technology [39]. The sequence for cDNA may immediately reveal point mutations and amino acid substitutions in the target enzyme that are responsible for the resistance to the new drugs [16, 40]. The amino acid substitution(s) can be analyzed using the known 3-dimensional structure for the target enzyme and interactive computer graphic display systems to reveal the mechanism for alteration of the binding of the drug to the active site of the enzyme. This information may reveal how the drug could be modified to yield a second ge-

neration drug for the treatment of the resistant strains of the pathogen.

This process does not need to await the appearance of resistant strains in the wild. Instead, drug resistant strains can be generated in pathogens cultured *in vitro*, under the pressure of low concentrations of the new drug. The development of resistance to the new drug can be analyzed as described above and mutations involving the target enzyme can be identified [41]. By this means, second generation drug can be designed to treat some of the possible resistant forms of a pathogen, even before they have occurred in the wild.

Closing. The promises of rational drug discovery/design have long been debated. However, technological advances have finally brought us to the point where enzyme structure based drug design can facilitate the development of new drugs for the treatment of a number of human maladies [8, 42—44].

С. Крег

ФЕРМЕНТИ ДОДАТКОВОГО ШЛЯХУ СИНТЕЗУ ПУРИНІВ — МІШЕНЬ ДЛЯ ХІМІОТЕРАПЕВТИЧНОГО ЛІКУВАННЯ ПАРАЗИТАРНИХ ХВОРОБ

Резюме

В огляді проаналізовано результати, які показують, що досягнення у вивченні специфічних молекул-мішеней клітини дозволяють розробляти ефективні лікарські засоби для лікування багатьох захворювань людини. Встановлено, що новий підхід до розробки таких медикаментів базується на дослідженні структури молекули фермента.

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08.06.94