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THE RATIONAL CHEMOTHERAPY OF CHAGAS' DISEASE: A POSSIBLE DREAM?

MOISES AGOSIN

THE PROBLEM

About 20 million Latin Americans are infected with *Trypanosoma cruzi*, the causative agent of Chagas' disease. In the chronic stages, patients may show evidence of lesions in the heart, esophagus and colon, which in many instances may lead to incapacitation or death. In spite of the serious public health hazard that Chagas' disease represents, treatment for chronic stage patients is not available, and drugs which are used with success against African trypanosomes and *Leishmania* sp have no effect on *T. cruzi*. Nifurtimox (Bayer 2502, Lampit) and benznidazole (RO-1051, Rodanil, Rochagan) are currently used in the treatment of acute Chagas' disease, but they have serious side effects and their ability to eradicate all parasites is in doubt (1).

The need for a safe and efficient chemotherapeutic drug for the treatment of *T. cruzi* infections cannot be overemphasized. Many drugs have been tested based on the empirical approach of the chemist to the problem of selective toxicity, but the long sought goal of a compound based on a more rational approach has eluded the many talented investigators involved in the experimental chemotherapy of Chagas' disease. The rational approach must be based on a through knowledge of the physiology and biochemistry of the parasite (2). Factors such as per-

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meability and transport mechanisms have to be considered and the metabolic pathways and individual enzymes operative in the parasite must be characterized and compared with those of the host in order to establish similarities and differences. The latter may be exploited for the design of chemotherapeutic drugs.

Molecular biology and rational chemotherapy

The spectacular advances made in the molecular biology area in recent years may provide a useful link between biochemistry and pharmacology to achieve the synthesis of drugs in the laboratory based on the rational approach. In a first stage, a suitable parasite enzyme would have to be selected. This could be either an enzyme unique to the parasite, a not so unique enzyme but with different substrate specificities, or an enzyme similar in many respects to the host one, but which may differ in turnover. Inhibition of a slowly turning over enzyme in the parasite would probably not affect the host if the latter's enzyme has a significantly higher turnover rate. In a second stage, the enzyme would be purified and monoclonal antibodies against the protein prepared. This would allow the preparation of the corresponding cDNA that when expressed in a suitable vector would provide sufficient amounts of pure protein for further studies. Sequential analysis of the enzyme protein would be deduced from the sequential analysis of the corresponding cDNA. As sufficient amounts of enzyme may be prepared by recombinant DNA techniques, crystallographic studies may be undertaken to establish the structure of the enzyme. On the basis of all the information obtained, computer analysis may lead to the design of a suitable enzyme inhibitor which may be synthesized in the laboratory. The inhibitor may be tested in *in vitro* systems and if it is effective, this may be followed by *in vivo* trials. This theoretical approach requires the participation of the parasitologist who has to maintain the parasite in animal transfers, and not only rear the organism in cultures but also obtain clones if required; the biochemist who has to do the enzymology work; the molecular biologist in charge of the recombinant DNA aspects; the chemical crystallographer and the synthetic organic chemist who will obtain the active compound and finally, the pharmacologist, who in collaboration with the parasitologist will conduct the drug trials. In the end, assuming positive results, physicians would be involved in trials in humans. Clearly, this is a multidisciplinary approach which requires sophistication but may be faster and less expensive than the usual trial and error approach.

We now will discuss examples in which some of the principles outlined above have been applied with some measure of success.

Pyrazolopyrimidines

Differences in the metabolism of purines between *T. cruzi* and the host have been utilized for the development of a rational approach to the chemotherapy of Chagas' disease (3, 5). *T. cruzi* does not synthesize purines *de novo* as does man. The parasite is able to concentrate pyrazolopyrimidines within the cell and metabolize them as purines through the salvage pathway ultimately incorporating them into nucleic acids. This does not occur in mammals. The pyrazolopyrimidine base allopurinol (4-hydroxypyrazolo [3, 4-α] pyrimidine, HPP) is activated by phosphoribosyl transferase to the ribonucleotide. The ribonucleotide is aminated to 4-aminopyrazolopyrimidine ribonucleotide and subsequently phosphorylated to the triphosphate form and incorporated into RNA. In man, about 60% is rapidly converted to oxipurinol, 30% is excreted in the urine, and the remaining 10% is converted to oxipurinol. 30% is excreted in the urine, and the remaining 10% is excreted as allopurinol-1-ribonucleoside (HPPR) (6). Besides HPPR, small amounts of 1-ribosyloxiourinol and 7-ribosyloxipurinol are formed through the action of phosphateses and the action of nucleoside phosphorylases (7, 8). No effects of allopurinol on purine metabolism in man have been noted (9).

The growth of *T. cruzi* epimastigotes is inhibited by HPP (3) *in vitro* and *T. cruzi* infected mice treated with allopurinol show significant increases in survival times when compared with controls (10, 11). However, some *T. cruzi* strains are not responsive to this type of compounds, which suggest that metabolic differences are present in these strains (3). This raises serious doubts as to the efficacy of these drugs to treat Chagas' disease in man. More recently, allopurinol ribonucleoside and another inosine analogue, formycin B have been tested against *Leihsmania* with some success (12, 13). However, evidence that formycin B cytotoxicity in *Leishmania* is not mediated by its incorporation as the adenoside analog into RNA has been presented (14). Instead, depletion of an essential intracellular metabolite is proposed for the action of this compound. In any event, it is clear that differences in the enzymology and metabolism of purines which exist in the genera *Trypanosoma* and *Leishmania* offer an opportunity for the design of chemotherapeutic drugs on a rational basis.

Cytochrome P-450 and antimycotic agents

Recently, antimycotic N-substituted azole derivatives which interfere at low concentrations with the microsomal 14 α-demethylase system have been found effective against *T. cruzi* amastigote infections in mice (15) and cultured peritoneal macrophages and fibroblasts (15, 16). This raises

the possibility that inhibition of enzyme systems involved in the biosynthesis of sterols in *T. cruzi* may be responsible for this chemotherapeutic effect.

The unsaponifiable lipid fraction of all parasites studied thus far contains a certain proportion of sterols (2). Cholesterol is the predominant compound in several protozoa such as *Trichomonoas, Entamoeba* and *Plasmodium*, but it is replaced by ergosterol in most trypanosomatids. Many of these parasitic sterols are of dietary origin and the composition of the environment will determine their nature or relative abundance. Thus, the bloodstream form of *T. brucei rhodesiensi* contains essentially cholesterol (17), and in *T. cruzi*, which does not accumulate cholesterol when grown in a cholesterol-free medium, it becomes the most abundant sterol when cholesterol is a component of the medium (18).

Very little is known about the ability of trypanosomes to synthesize sterols. *De novo* synthesis has been observed only in some trypanosomatids. Thus, *Crithidia fasciculata* incorporates labeled carbon from acetate, mevalonate and methionine into ergosterol (19). The bloodstream form of *T. lewisi* as well as the culture form of *T. brucei rhodesiense* synthesize sterols from acetate, but their identity has not been yet proved (20).

Neutral lipid extracts of *T. cruzi* epimastigotes show an ultraviolet spectrum typical of the presence of 5, 7-diene sterols, and chromatographic analysis has revealed the presence of cholesterol, ergosterol, 24-methylene-7-dehydrocholesterol and 7-dehydroporiferasterol (21). It was concluded that 5, 7-diene sterols represent about 93% of total epimastigote sterol, and that ergosterol is an important component of the parasite membrane, as reported for *C. fasciculata* sterols (22). A more detailed analysis of *T. cruzi* sterols by gas chromatography/mass spectrometry (23) has confirmed the presence of fungal-type sterols in the organism. Cholesterol from the medium and several endogenous C28 and C29-4-desmethyl sterols which had been tentatively identified some years ago in Von Brand's laboratory (18) were detected. Ninety per cent of the free sterols corresponded to the 4-desmethyl sterols and 8% to 4,4-dimethyl sterols which included some lanosterol and 24-methyllanosta-8, 24 (28)-diene-3 β-ol. The epimastigotes showed the *de novo* synthesis of sterols as evidenced by the incorporation of (2-14C)-mevalonate into the 4-desmethyl sterols and the 4, 4-dimethyl sterol fractions (23). The latter observation suggests that pathways for the synthesis of sterols in mammals, fungi and yeast may be operative in *T. cruzi*.

THE EFFECT OF ANTIFUGAL COMPOUNDS ON *T. CRUZI*

Certain heterocycle-containing antifungals as well as imidazole and triazole antifungals are known to inhibit the biosynthesis of ergosterol (24). It is established that ergosterol is a critical component of microsomal and mitochondrial membranes and its depletion alters the physicochemical properties of these membranes. Azole derivates such as Ketoconazole inhibit sterol synthesis in *Candida albicans* at concentrations as low as 5 nM (24). The inhibition of ergosterol biosynthesis by these antifungals correlates with the accumulation of 14 α-methylated sterols, which is indicative of interference with the cytochrome P-450 14 α-demethylase system. Yeast cytochrome P-450 catalyzing lanosterol 14 α-demethylation has been purified and characterized (25, 26). Similar effects have been found in *T. cruzi*. Thus, miconazole and econazole, two fungicide imidazole derivatives produce a decrease of 5, 7-diene sterols, including ergosterol. This effect is observed at concentrations of about 4 to 10 μM and is paralleled by profound ultrastructural alterations (21). Ketoconazole at concentrations 100-fold lower than miconazole and econazole produces a decreases in cholesterol and the endogenous desmethyl sterols, coincident with the accumulation of lanosterol and 24-methylene dihydrolanosterol (23). After 5 days of incubation, 77% of the total free sterols of epimastigotes corresponded to 4, 4, 14 α-trimethyl sterols and 92% of these was 24-methylene dihydro lanosterol, which is the same product which accumulates in *C. albicans* exposed to ketoconazole (27). These effects were accompanied by inhibition of the parasite growth at concentrations as low as 20 nM. The loss os cholesterol is apparently due to the impairment of membrane-associated mechanisms of cholesterol uptake, secondary to the changes in membrane lipid bilayersterol composition. Cytochrome P-450 species have been described in *T. cruzi* epimastigotes as well as trypomastigotes (28).

On the basis of the rather fragmentary information available on the biosynthesis of sterols in *T. cruzi*, the nature of the sterols found (21, 23) suggests that similar pathways to those leading to the synthesis of cholesterol from lanosterol in mammals may be operative in the parasite. In mammals, the conversion of lanosterol to cholesterol is a complex process which involves reduction of the Δ^8-bond of lanosterol to the Δ^5-bond location of cholesterol. The latter process is catalyzed by the Δ 7-sterol-5- desaturase; whereas C-4 demethylation is carried out by methyl sterol oxidase. Both enzyme systems require cytochrome b5 as electron carrier, they are membrane-bound, and because of their sensitivity to inhibitors, appear to be analogous to other cytochrome b5-

dependent oxidases such as the fatty acyl CoA desaturase (28), phospholipid desaturase (29), and a terminal oxidase in the microsomal fatty acid elongation system (30). Recently, the Δ^7-sterol-5-desaturase of rat liver microsomes has been purified and characterized (31). None of these enzymes have been investigated in *T. cruzi*, however, the 14 α-demethylation reaction catalyzed by cytochrome P-450 clearly occurs in the parasite, as shown by the type of sterols which accumulate under the effect of ketoconazole (23). *T. cruzi* epimastigotes contain at least 2 cytochrome P-450 species (3) but whether any of these forms is responsible for the 14 α-demethylation reaction remains to be demostrated. The fact that 4, 4, 14 α-trimethyl sterols accumulate in epimastigotes treated with ketoconazole (23) suggests that in addition to 14 α-demethylation, other reactions of sterol biosynthesis may also be impaired.

Some undesirable effects of antifungals

It has been reported that the effect of ketoconazole on yeast microsomal cytochrome P-450 is selective (34) because higher concentrations are required to affect rat liver cytochrome P-450. Nevertheless, the compound inhibits testosterone synthesis in subcellular fractions of rat testis, possibly through interaction with cytochrome $P\text{-}450_{scc}$. This effect is seen at concentrations higher than those required to inhibit growth of *T. cruzi* (24). Itraconazole, on the other hand does not show this effect even at concentrations as high as 10^{-5}M. Ketoconazole, miconazole and clotrima zole are inhibitors of progesterone 16 α-and 17 α-hydroxylase activities which are cytochrome P-450-dependent reactions, and other antimycotic agents are potent inhibitors of steroid aromatase activity of human placenta microsomes (35). The above observations suggest that the use of antimycotic agents in the chemotherapy of Chagas' disease may be somewhat premature and that further biochemical studies are required for the proper design of specific inhibitors of the enzyme systems involved. Nevertheless, the introduction of these antifungals as well as allopurinol derivatives represent a milestone in the development of chemotherapeutic compounds based on either differences in the basic metabolism and/or drug metabolizing capabilities of host and parasite and a remarkable example of the rational approach to chemotherapy. Hopefully, application of the principles outlind in this overview may provide more efficient and selective compounds for the treatment of this important disease.

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