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Ribonucleotide reductase as target for drug discovery against African sleeping sickness

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Introduction

African sleeping sickness is a debilitating disease for which about 300,000 new cases are reported annually in some developing African countries south of the Sahara where about 60 million people in some 200 locations are exposed to the risk if infection [1]. The disease, also called trypanosomiasis, is a problem to man and domestic animals in Africa as several areas of the disease process are yet to be understood.

A major feature of trypanosomiasis is that upon infection there is parasite proliferation for the trypanosome species to establish its population in the infected host and this emanates from binary cell division. Controlling the process of binary cell division in the bloodstream forms of the parasite could control parasite proliferation and parasitaemia which correlates with severity of infection.

The present situation is such that treatment of the disease with few available old and often expensive drug types is unsatisfactory [2,3] thereby creating the need to identify new molecular mechanisms and targets against which new drugs could be developed. Ribonucleotide reductase (RNR) is a highly researched enzyme for anticancer therapy [4]. The central role of the enzyme in providing the deoxyribonucleotide (dNTP) precursors in DNA replication during the cell division cycle and the subsequent cell proliferation has made it a desirable target for drug design [4,5]. It has in recent times been receiving attention as a useful target for antiparasite therapies [5]. This review seeks to update the status of this enzyme in African trypanosomes and highlight the several ways by which it can be exploited to develop or determine potential drugs against African trypanosomiasis. It is quite surprising that most reviews including recent ones [2,3] on control strategies for trypanosomiasis hardly mention this enzyme.

Structural aspects of RNR

The structure or RNR [6] for which there are essentially three classes [6,7] has been aptly described in very few words in a recent publication [8]. The classes are Ia, Ib, II and III. Most eukaryotic cells and a few prokaryotes contain a class Ia enzyme. It has been suggested that the enzyme in the African trypanosome, *T. brucei* is of class Ia [9]. The functional class Ia enzyme is composed of two non-identical homodimeric subunits, R1 and R2, both of which are individually inactive. The larger subunit (R1) contains two α-polypeptides while R2 contains two β-polypeptides. Each of the larger α-polypeptides contains redox active disulphides, an active site that binds ribonucleoside diphosphates, and two different allosteric sites that bind nucleoside triphosphates. One allosteric site regulates the overall enzyme activity by binding ATP for stimulation and dATP for inhibition. The other allosteric site controls substrate specificity by binding ATP, dATP, dTTP or dGTP. In the smaller R2 subunit, the two β-polypeptides

together create an iron tyrosyl free radical centre for activating the enzyme substrates. The highly flexible carboxyl terminal tail of the β-polypeptide is esential for the formation of the $\alpha_2\beta_2$ heterotetramer, the rigid complex that becomes the active enzyme [6,8].

The *T. brucei* enzyme olaced among the class Ia RNRs shows amino acid sequence homology to mouse RNR and possesses iron-tyrosyl free radical centre as well as R2 protein carboxyl terminal tail for binding to R1 protein [9]. The reduction of CDP by the trypanosome enzyme is however not regulated by dATP [9,10], a feature also not demonstrated by some other organisms in classes Ia, Ib and II [7].

Regulatory aspects of RNR

The structural considerations of the enzyme in relation to function provide the basis for the regulation and subsequent exploitation of the enzyme for parasite killing and disease control. The enzyme activity can naturally be regulated at the protein level and also at the transcriptional or translational levels. These are also exploitable levels of enzyme inhibition or drug development against the parasite, putting species differences between the parasite and the host into consideration.

Where there are no species differences, a reasonable factor for consideration is the multiplication rate between the trypanosome or other parasitic cells and the host cell. African trypanosomes multiply at a rate much faster than the mammalian host cells [11] and are therefore likely to be more susceptible to single or combined inhibitors of cell division including those that regulate the cell division cycle-dependent RNR activity, directly or indirectly. Another fortunate thing is that it has not been established beyond doubt that African trypanosomes can penetrate and lodge in any of the host cell types. In other words, each cell type can individually take up inhibitors or drugs at its own rate. Interestingly, RNR has for a long time remained a target enzyme for anticancer therapy and the inhibitors of the enzyme have been widely reported [4,12,13].

At the protein level, the RNR enzyme can be inhibited by substrate analogues [14-16], tyrosyl radical scavengers [4,17]' peptidomimetic substabces [18-20], iron deprivation [21] and, as recently reported [22,23], by a novel protein labelled Sm11. It is expected that RNR activity could also be regulated by the inhibitors of the thioredoxin and glutaredoxin systems which deliver reducing equivalents to RNR for its activity [24]. The emerging picture is that the "tryparedoxin" system is present in African trypanosomes as the equivalent system that delivers electron to the parasite RNR [25-29].

RNR shows the cell cycle phase-specific expresion [30] which therefore makes it of considerable interest to study transcriptional control mechanisms for the R1 and R2 genes. The promoter activities including the transcription factors for the genes could be highly informative for drug discovery and development. Such promoter and expression analyses have been done for some microbes [31], human [32-34] and mouse [30, 35-37] enzymes. Identifying and characterising the expression mechanism for trypanosome RNR could provide opportunities for trypanocide discovery or development. The genes for trypanosome RNR have been cloned, expressed and characterised [9,38] but the promoter activities, including transcription factors for the genes have not been identified and characterised. Perhaps the real regulation is post-transcriptional as most protein-coding genes in African trypanosomes are organised in polycistronic rather than monocistronic transcription units and co-transcribed from a common promoter [39]. In African trypanosomes, the regulation for the expression of the genes for RNR may as well be in the pathway between transciption and translation as the R2 subunit has been suggested to be post-transcriptionally regulated [40].

Possibility for trypanosome RNR inhibition

RNR is the rate-limiting enzyme of *de novo* synthesis of deoxyribonucleotides. It shows increased activity in proliferating cells [41,42]. Apart from the high multiplication rate of trypanosomes when compared with the rate for the mammalian host cells, subtle differences in the properties of the parasite and mammalian enzymes present another line of exploitable opportunities for chemotherapy.

Substrate analogues

The report on allosteric regulation of the trypanosome RNR [10] with clear differences from mammalian enzymes provides a basis for possible inhibitory substrate analogues. It is noteworthy that uncontrollable uptake of deoxyadenosine strongly inhibited trypanosome proliferation [10]. It is also worth noting that

CDP and CTP levels are extremely low in *T. brucei* while dCTP pool is comparable with the relative levels in other organismz [10]. In the light of these considerations, deoxyadenine and deoxyadenosine analogues which show cytotoxic effects by inhibiting RNR [14,16] could be posible trypanocides. The derivatives of deoxycytidine such as gemcitabine (dFdC), (E)-2-deoxy-2-(fluoromethylene) cytidine (FMdC or ML 101,731) and especially 2-deoxy-2-methylidenecytidine (DMDC) could also be possible trypanocides as they have been reported to show antitumour activity and to inhibit RNR [15,43].

Hydroxyurea

Each of the smaller R2 subunits contributes iron centre-generated tyrosyl free radical to the active enzyme. Destabilisation of the iron centre inactivates the enzyme. A number of compounds have been identified which inhibit RNR by this mechanism [44] but hydroxyurea is the only one that is currently clinically in use as anti-cancer agent [17]. Hydroxyurea has already been reported to control trypanosome growth *in vitro* [10,45] and to inhibit trypanosome purified RNR [10]. The potential limitations for hydroxyurea could however be the relatively low affinity of RNR for it [46], short half life in humans [46] and relative ease with which resistance develops to it [44]. Hydroxyurea may however be combined with other possible inhibitors. Dideoxyinosine, for instance, shows synergism with hydroxyurea [47].

Protein-protein interaction

The C-terminal tail of the β-polypeeptides, through which R1 and R2 form the active enzyme complex, is another point at which anti-trypanosomal drugs could be could be speculated. It has been reported that the IC_{50} of a heptapeptide corresponding to the mouse R2 C-terminus was similar for both mouse and *T. brucei* RNR [9]. This observation was attributed to the almost identical amino acids sequences in both enzymes. The lack of interspecies interaction between R1 and R2 of the enzymes from both sources [9] however suggests subtle but significant differences in the mammalian and trypanosome R2 C-terminal sequences. These considerations suggest further studies with different types of peptidomimetic inhibitors as possible trypanocides. Several peptidomimetic inhibitors of RNR have been reported [18-20].

The Sm11-mediated negative control of RNR is also at the protein level [2,23] specifically building to the R1 protein [22] and inhibiting the synthesis of deoxyribonucleotides. Mec1 and Rad53 proteins are required to remove the inhibitory effect of Sm11 on RNR during S phase of the cell cycle to facilitate the production of deoxyribonucleotides for DNA replication [23]. This implies that cell division and proliferation could be regulated by blockers of Mec1 and Rad53. The Sm11-mediated control is yet to be studied in African trypanosomes.

Iron chelators

Iron chelation therapy is gradually gaining grounds as a strategy for the control of microbial infections, especially malaria [49,50]. This kind of therapy is yet to receive serious attention with respect to African trypanosomiasis. There are unpublished indications that iron chelation could be a potent means of killing African trypanosomes *in vitro* and *in vivo*. RNR is suspected to be the prime target. The enzyme is the only one along the cell division cycle that requires iron, not as a prosthetic group, but as part of the free iron pool [51,52]. The recombinant RNR enzyme of the trypanosome contains iron-tyrosyl free radical centre [9] thus suggesting that the native enzyme could be subjected to regulation by iron chelators. RNR is inhibited by iron chelators [21,52] and Triapine has been described as being a superior anticancer agent over hydroxyurea [12].

Transcriptional control

Development of drugs against African sleeping sickness through the inhibition of the RNR at the transcriptional level is not yet in sight. The regulatory sequences together with possible regulatory factors for trypanosome RNR transcriptional or post-transcriptional control have not been determined. An initial step towards this, however, is the report by Breidback *et al*. [40] suggesting that R2 is post-transcriptionally regulated.

Concluding Remarks

African trypanosomiasis continues to suffer inadequate attention considering the next-to-lack of introduction of new, effective, non-toxic and affordable drugs for almost half a century. It is speculated that some of the old drugs may not pass today's safety test [53]. The recently introduced eflornithine also has its own limitations and difficulties in application in the typical rural African setting [3]. A principal problem in introducing new trypanocides, however, relates to economics [54] as the pharmaceutical industry cannot profitably justify the expenses needed for the development of novel reagents against the disease of the very poor Africans who cannot afford to pay for the end result. One suggestion to surmount this is to develop new compounds which are active against diseases prevalent in rich countries and, at the same time, active against African trypanosomiasis [2]. The inhibitors of RNR activity could fall into this category and they may be very useful in the very near future if research efforts are concentrated on trypanosome enzymes as target. The array of anticancer agents targeting RNR [4], some of which are already in clinical use, provide a good point to start. Considering the problems associated with the current therapies of African sleeping sickness which include toxicity, resistance and economics, it is suggested that focus should shift to the trypanosome RNR which recent reviews on chemotherapy of the disease [2.,3] fail to address. From among the inhibitors of the enzyme, iron chelation therapy with drugs already in clinical use against iron toxicity or as anticancer agents is highly favoured.

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