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Resistance of schistosomes to hycanthone and oxamniquine

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Summary: Genetic crosses between phenotypically resistant and sensitive schistosomes demonstrated that resistance to hycanthone and oxamniquine behaves like a recessive trait, thus suggesting that resistance is due to the lack of some factor. We hypothesized that, in order to kill schistosomes, hycanthone and oxamniquine need to be converted into an active metabolite by some parasite enzyme which, if inactive, results in drug resistance. Esterification of the drugs seemed to be the most likely event as it would lead to the production of an alkylating agent upon dissociation of the ester. An artificial ester of hycanthone was indeed active even in resistant worms, thus indirectly supporting our hypothesis. In addition, several lines of evidence demonstrated that exposure to hycanthone and oxamniquine results in alkylation of worm macromolecules. Thus, radioactive drugs formed covalent bonds with the DNA of sensitive (but not of resistant) schistosomes; an antiserum raised against hycanthone detected the presence of the drug in the purified DNA fraction of sensitive (but not of resistant) schistosomes; a drug-DNA adduct was isolated from hycanthone-treated worms and fully characterized as hycanthone-deoxyguanosine.

In 1971 Rogers and Bueding[1] first reported the occurrence in the laboratory of schistosomes which were genetically resistant to hycanthone. These results were later confirmed by additional work of the same group[2], but were found not to be applicable to all schistosome strains[3]. In 1973, Katz and coworkers[4] reported the occurrence of drug resistance in schistosome strains isolated from treated patients and in subsequent years this was followed by similar reports from Brazil[5,6,7,8] and from Kenya[9].

A general feature emerging from these studies is that hycanthone resistant schistosomes are also resistant to oxamniquine, while, as a rule, they are sensitive to different antischistosomal drugs. This is in accordance with the structural similarities between hycanthone and oxamniquine: they both have an alkylamino-ethylamino side chain in *para* position with respect to a hydroxymethyl group which is critical for activity and they both are oxidative metabolites of parent compounds possessing a methyl group in place of the hydroxymethyl group. The biological and chemical similarities between the two drugs strongly suggest that they have a similar mechanism of action[10] and the experimental evidence to be reported here lends support to this contention.

The term "resistance" is usually defined as a genetically transmitted loss of sensitivity in a population which was previously sensitive to a given drug, while the lack of sensitivity in a previously untested population should be more correctly termed "tolerance". Another distinction is usually made between resistance "induced" and resistance "selected" by a given drug. These distinctions may have some theoretical value, but from the practical standpoint resistance and tolerance pose the same clinical problems, while the direct "induction" of resistance by a drug is probably a very rare event. The driving forces of evolution, i.e. *mutation* and *selection* are also the key events in the production of resistance to a given drug, but <u>neither</u> of these events is necessarily connected with the use of that drug. The more general term "resistance" will be used here without a specific reference to the distinctions mentioned above.

Two separate, but strictly interconnected, issues will be considered, i.e. the mechanism by which hycanthone and oxamniquine exert their schistosomicidal activity and the mechanism by which some schistosome strains are able to escape such activity. An answer to the first set of problems can be given with a fair amount of confidence, while only indirect evidence exists regarding the second type of problems.

<u>Resistance to hycanthone and oxamniquine is a recessive trait.</u>

In some biological systems drug resistance results from the acquisition of a new activity in resistant organisms (e.g. the ability to degrade and inactivate the drug), while in other systems resistance results from the loss of a pre-existing activity (e.g. the loss of drug-activating mechanisms). We first attempted to classify hycanthone/oxamniquine resistance into one of these two broad categories by performing schistosome genetic crosses aimed at determining whether resistance was a dominant or recessive trait. A dominant character usually results from the expression of a gene which, even in a single copy, is able to confer a given phenotype; a recessive character requires that both copies of a gene be mutated (e.g. inactive) in order to obtain the mutant phenotype. Thus, a dominant mutation is usually connected with the acquisition of a new activity, while a recessive mutation is usually connected with the loss of some activity.

The schistosome strain which is currently used in our laboratory is sensitive to hycanthone, since less than 10% of the parasite survive an *in vivo* treatment with 80 mg/Kg to the mouse host or an *in vitro* treatment with 10^{-6} M drug in the medium. In addition, repeated attempts have been made to select hypothetical resistant mutants out of

this strain, but the result was always negative, as experienced by others with a number of schistosome strains[3]. Thus, we interpret these data as a strong indication that our schistosome population is uniformly sensitive to hycanthone and, since no resistant individuals were ever found, we presume that the strain is also homozygous for whatever gene(s) control the sensitive phenotype. Our resistant schistosomes belong to a different strain which was originally selected in Bueding's laboratory for its resistance to hycanthone. In addition, we applied further selective pressure to this strain by subjecting infected mice to drug treatment for 3 subsequent generations; thus, only the resistant survivors had a chance of reproducing. Again, this resistant strain appears to be very homogeneous (and probably homozygous for resistance), since infected mice never showed any decrease in egg output after treatment and *in vitro* cultured adults had a 100% survival after exposure to 10^{-4} M hycanthone.

The procedure we adopted to perform genetic crosses was greatly simplified with respect to the traditional protocol involving single-sex infections. We simply transplanted one (or more) 27-day old male from one strain and one (or more) female from the other strain into uninfected mice, using an adaptation of the transfer technique originally described for hamsters[11]. About 3-4 weeks later hybrid eggs were available to infect snails. The procedure adopted to assess drug resistance/sensitivity consisted initially in infecting two groups of mice and subsequently treating one group in order to determine the percent of survivors (resistant). The results obtained using this procedure have been previously reported[12]. A more directly quantitative approach has been recently introduced, which consists in culturing schistosomes after *in vitro* drug exposure. This permits a direct enumeration of parasites which die or survive after 2-3 weeks in culture. The results obtained with the *in vitro* procedure are in good general agreement with the results obtained *in vivo* and are partially summarized in Table I.

Table I. Results of genetic crosses between hycanthone sensitive and hycanthone resistant schistosomes. Values represent the number of adult schistosomes analyzed *in vitro*. S = sensitive strain ; R = resistant strain.

Parents ♂	Parents ♀	♂ Progeny Examined	♂ Progeny Resistant	♂ Progeny % Resistant	♀ Progeny Examined	♀ Progeny Resistant	♀ Progeny % Resistant
<u>F_1 generation</u>							
S	S	112	0	0	112	0	0
R	R	162	162	100	162	162	100
S	R	112	0	0	112	0	0
R	S	250	0	0	186	0	0
<u>F_2 generation</u>							
(SR)	(SR)	990	198	20.0	626	147	23.5
(RS)	(RS)	188	41	21.8	182	33	18.1

From the results of the F_1 and F_2 generation it can be concluded that resistance is controlled by a recessive autosomal gene. The frequency of resistant schistosomes in the F_2 generation is sometimes slightly but significantly lower than the expected 25% and the reasons for this discrepancy are currently under investigation.

Metabolic drug activation seems to be required for antischistosomal activity.

The evidence from genetic crosses suggests that resistance is due to the absence of some factor, whereas this factor is present (at least from a single gene copy) in phenotypically sensitive schistosomes. In other words, hycanthone needs some worm activity in order to exert its schistosomicidal effects. One simple interpretation is that a metabolic drug activation may occur in sensitive schistosomes. By considering the mode of action of hycanthone and oxamniquine, we had previously suggested that a covalent bond between the drug and some critical parasite macromolecule was probably involved in the antischistosomal effects, since a very short contact (minutes) between drug and parasites was an irreversible event which brought about schistosome death several days later[13]. One possible mechanism by which the drug could be converted into an alkylating agent is represented by drug esterification followed by ester dissociation. This would convert the drug into a charged moiety which could easily alkylate parasite macromolecules.

We decided to attempt a preliminary test of the above hypothesis by synthesizing an artificial model ester of hycanthone, i.e. hycanthone N-methyl carbamate. This ester was tested both in vitro and in vivo and it was found that it had roughly the same antischistosomal activity as the parent compound, but had the important new characteristic of being active against both sensitive and resistant schistosomes[14]. Several different hycanthone esters were synthesized and a number of them were also active against resistant worms[15]. Thus, if the need for metabolic activation is bypassed by exposing schistosomes to a preformed ester, worms of the resistant strain cannot escape alkylation and death.

This type of evidence gives strong, but indirect, support to our hypothesis about the mechanism of drug action and the mechanism of drug resistance. It must be added, however, that when similar esters of oxamniquine were synthesized, they failed to show any activity against resistant worms. We do not know what is the actual ester (if any) which is produced in the schistosome; we were unable to assay the most likely candidates, i.e. the phosphate and the sulfate, since they were too unstable. Thus, it is possible that we are not testing the appropriate esters of oxamniquine, but the lack of parallelism with hycanthone is a finding which we cannot presently explain on the basis of our general hypothesis.

Hycanthone and oxamniquine alkylate the DNA of sensitive, but not of resistant, worms.

Further evidence for the mechanism of action of hycanthone and oxamniquine was obtained through the use of radioactively labeled drugs. Sensitive and resistant schistosomes were separately incubated in vitro with tritiated hycanthone and the radioactivity was determined in the total worm homogenate and in isolated worm fractions. Total radioactivity at short times after drug exposure was very similar in sensitive and resistant worms, thus suggesting that resistance is not due to different drug uptake by the two strains. Radioactivity in purified macromolecular fractions, however, was much higher in sensitive worms than in resistant ones. DNA-bound hycanthone was almost

negligible in resistant worms, while it reached very high levels (about 1 molecule every 50,000 base pairs) in sensitive schistosomes[16]. Although proteins and RNA of sensitive worms also bound large amounts of drug, we concentrated our attention on the DNA fraction since previous studies from our laboratory had suggested that DNA synthesis was probably affected in hycanthone activity[17,18].

We also incubated sensitive and resistant schistosomes with tritiated hycanthone N-methylcarbamate and in this case the amount of radioactive drug bound to the DNA of resistant worms was similar to the amount bound to the DNA of sensitive worms, as predicted by our hypothesis[16].

Radioactive oxamniquine was also synthesized and it was found that it bound in large amounts to the DNA of sensitive worms, while the DNA of resistant worms had levels of radioactivity which were at or below our detection threshold[19]. It was also found that female worms, which are less sensitive to the action of oxamniquine, bound less radioactive drug to the DNA than their male counterpart.

Evidence for selective binding of hycanthone to the DNA of sensitive, but not of resistant, worms was also obtained using a completely different approach which was not dependent on the use of radioactive drug. A rabbit antiserum was obtained which was capable of recognizing hycanthone. This antiserum was used to test the DNA isolated from sensitive and resistant worms exposed to non-radioactive hycanthone. The results showed again the presence of hycanthone in the DNA of sensitive worms and its absence in the DNA of resistant schistosomes (Pica-Mattoccia and Cioli, unpublished results).

Finally, we asked which specific target in the DNA was alkylated by hycanthone. An hycanthone-deoxyguanosine adduct was synthesized and it was shown to co-elute in HPLC with the major radioactivity peak obtained from a digest of the DNA of sensitive schistosomes incubated with radioactive hycanthone (unpublished results). Thus, the major site of alkylation in the DNA appears to be deoxyguanosine.

Conclusion

Evidence presented here indicates that hycanthone and oxamniquine have a similar mechanism of antischistosomal activity, the end-result being the formation of a covalent bond between drug and parasite macromolecules, notably the DNA. We also presented some indirect evidence showing that an intermediate step in the drug mode of action may be its conversion to an active ester which, upon dissociation, yields an alkylating agent. We suggest that the difference between sensitive and resistant schistosomes may be the absence in the latter strain of a drug-esterifying activity. A direct demonstration of our hypothesis would involve the isolation of the postulated esterifying enzyme from sensitive worms, while the corresponding activity should be absent in resistant worms. Work is in progress along these lines.

References

1. Rogers,S.H. and Bueding,E. - Hycanthone resistance: Development in *Schistosoma mansoni*. *Science*, **172**: 1057-1058, 1971.
2. Jansma,W.B., Rogers,S.H., Liu,C.L. and Bueding,E. - Experimentally produced resistance of *Schistosoma mansoni* to hycanthone. *Am.J.Trop.Med.Hyg.*, **26**: 926-936, 1977.
3 Yarinsky,A. - Susceptibility of schistosomes to chemotherapy with particular reference to hycanthone. *Proc.Int.Conf.Schisto.*, **1**: 259-269, 1978.
4. Katz,N, Dias,E.P., Araujo,N and Souza,C.P. - Estudo de uma cepa humana de *Schistosoma mansoni* resistente a agentes esquistossomicidas. *Rev.Soc.Bras.Med.Trop.*, 7: 381-387, 1973.
5. Campos,R., Moreira,A.A.B., Sette,H., Chamone,D.A.F. and Da Silva,L.C. - Hycanthone resistance in a human strain of *Schistosoma mansoni*. *Trans.Roy.Soc.Trop.Med.Hyg.*, **70**: 261-262, 1976.
6. Guimarães,R.X., Tchakerian,A., Dias,L.C.S., de Almeida,F.M.R., Vilela,M.P., Cabeça,M. and Takeda,A.K. - Resistência ao hycanthone e oxamniquine em doentes com esquistossomose forma clínica hepatintestinal. *Rev.Ass.Med.Brasil*, **25**: 48-50, 1979.
7. Dias,L.C.S., Pedro, R.J., Rigo,E., Goto,M.M.F. and Mafra,G.L. - Linhagem humana de *Schistosoma mansoni* resistente a esquistossomicidas. *Rev.Saúde Públ.São Paulo*, **12**: 110, 1978.
8. Dias,L.C.S., Pedro,R.J. and Deberaldini,E.R. - Use of praziquantel in patients with schistosomiasis mansoni previously treated with oxamniquine and/or hycanthone: resistance of *Schistosoma mansoni* to schistosomicidal agents. *Trans.Roy.Soc.Trop.Med.Hyg.*, **76**: 652-659, 1982.
9. Coles,G.C., Mutahi,W.T., Kinoti,G.K., Bruce,J.I. and Katz,N. - Tolerance of Kenyan *Schistosoma mansoni* to oxamniquine. *Trans.Roy.Soc.Trop.Med.Hyg.*, **81**: 782-785, 1987.
10. Pica-Mattoccia,L. and Cioli,D. - Studies on the mode of action of oxamniquine and related schistosomicidal drugs. *Am.J.Trop.Med.Hyg.*, **34**: 112-118, 1985.
11. Cioli,D. - Transfer of *Schistosoma mansoni* into the mesenteric veins of hamsters. *Int.J.Parasitol.*, **6**: 355-362, 1976.
12. Cioli,D. and Pica-Mattoccia,L. - Genetic analysis of hycanthone resistance in *Schistosoma mansoni*. *Am.J.Trop.Med.Hyg.*, **33**: 80-88, 1984.
13. Cioli,D. and Knopf,P.M. - A study of the mode of action of hycanthone against *Schistosoma mansoni* in vivo and in vitro. *Am.J.Trop.Med.Hyg.*, **29**: 220-226, 1980.
14. Cioli,D., Pica-Mattoccia,L., Rosenberg,S. and Archer,S. - Evidence for the mode of antischistosomal action of hycanthone. *Life Sci.*, **37**: 161-167, 1985.
15. Archer,S., Pica-Mattoccia,L., Cioli,D., Seyed-Mozaffari,A. and Zayed,A. - Preparation and antischistosomal and antitumor activity of hycanthone and some of its congeners. Evidence for the mode of action of hycanthone., *J. Med. Chem.*, **31**: 254-260, 1988.
16. Pica-Mattoccia,L., Cioli,D. and Archer,S. - Binding of tritiated hycanthone and hycanthone N-methylcarbamate to macromolecules of drug-sensitive and drug-resistant schistosomes., *Mol. Biochem. Parasitol.*, **31**: 87-96, 1988.

17. Pica-Mattoccia,L., Lelli,A. and Cioli,D. - Effect of hycanthone on *Schistosoma mansoni* macromolecular synthesis in vitro. *Mol. Biochem. Parasitol.*, *2*: 295-307, 1981.
18. Pica-Mattoccia,L. and Cioli,D. - Effect of hycanthone administered in vivo upon the incorporation of radioactive precursors into macromolecules of *Schistosoma mansoni.*, *Mol. Biochem. Parasitol.*, **8**: 99-107, 1983.
19. Pica-Mattoccia,L., Cioli,D. and Archer,S. - Binding of oxamniquine to the DNA of schistosomes., *Trans. Roy. Soc. Trop. Med. Hyg.*, **83**: in press.