ADVANCES IN

Pharmacology and Chemotherapy

EDITED BY

Silvio Garattini
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I. J. Kopin

Consulting Editor

R. J. Schnitzer

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I. Introduction

Schistosomiasis, a world-wide parasitic disease, affects more than 200 million people, about 70 million of them infected with *Schistosoma mansoni*.

Although the introduction of the first antischistosomal agent occurred more than five decades ago (Christopherson, 1918) and despite the efforts expended in the search for active compounds—according to Standen (1967) more than 250,000 chemical substances have already been tested—few drugs have qualified for clinical trial and can be considered as antischistosomal agents of proven value.*

The various prerequisites for an ideal antischistosomal agent have been discussed by Fairley (1951), Newsome (1962b), Friedheim (1967), Pellegrino and Katz (1968). With some modifications, they can be summarized as follows: (*1*) absence of side effects and toxicity in man; (*2*) high activity against the three main human schistosome infections; (*3*) efficient when given in a single dose or, at most, for 2 days; (*4*) equally effective by injection or by the oral route; (*5*) active against all stages of the schistosome in mammalian hosts; (*6*) chemically stable under common storage conditions; and (*7*) low priced. However, as has been pointed out by Newsome (1962b), this ideal drug represents an aim to be achieved and serves as a criterion for the evaluation of the results so far obtained and those yet to come. A less ambitious and more realistic approach to the problem of chemotherapy of schistosomiasis is to consider that three types of drugs are necessary; (*a*) prophylactic, to prevent infection; (*b*) suppressant, to prevent egg laying; and (*c*) curative, to kill all or most of the adult worms.

So far, the compounds routinely used in clinical treatment, namely, antimonials, hycanthone, niridazole, and oxamniquine fulfill only some of the prerequisites mentioned.

Three approaches have been followed in the search for new drugs for

* General reviews on chemotherapy of schistosomiasis have been published, among others, by Standen (1963), Lämmler (1968), Pellegrino and Katz (1968), Werbel (1970), Archer and Yarinsky (1972), Friedheim (1973), Katz and Pellegrino (1974a). A very extensive bibliography of the world literature about schistosomiasis, from 1852 to 1962, can be found in Warren and Newill (1967). The present review contains references up to and including 1975.

the chemotherapy of schistosomiasis: the empirical, the selective, and the biochemical.

The empirical approach consists of blind screening a large number of compounds from chemically unrelated groups. The substances are tested in a standardized way in the hope that one or more of them may display activity against the infection and be, therefore, useful as a chemical "lead." This is the most usual approach in large-scale experimental chemotherapy. Once the lead is in hand, the active group becomes the focus of the selective approach.

The selective approach is the biological investigation of compounds chemically related to those already known to have some antischistosomal activity. The principal aim is to increase activity and/or decrease toxicity through structure modifications of that parent compound. Two drugs, at least, have recently emerged from this approach: hycanthone, from Miracil D (Rosi *et al.*, 1965), and oxamniquine, from the mirasan series (Richards and Foster, 1969).

The biochemical approach [also called rational approach (Standen, 1967)] is based on the chemical differences between the metabolic pathways and enzymes in the *S. mansoni* worm and in its host. Theoretically, this would be the best approach, since the drug could interfere with some vital system in the worm, without exhibiting toxicity to the host. However, up to the present, too little is known of the parasite's biochemistry to permit such forecasting. During the last decades, basic studies of the biochemistry and physiology of schistosomes have been carried out, especially by Bueding and co-workers, Senft, and others. These papers will not be reviewed here, and those interested in further details may refer to the articles of Bueding *et al.* (1947, 1953), Bueding (1949, 1950, 1952, 1959, 1962, 1967, 1969), Bueding and Koletsky (1950), Bueding and Charms (1951), Bueding and Peters (1951), Mansour and Bueding (1953, 1954); Mansour *et al.* (1954), Bueding and MacKinnon (1955a,b), Bueding and Mansour (1957), Timms and Bueding (1959), Senft (1963, 1965, 1966), Barker *et al.* (1966), Fripp (1967a,b), Booth and Schulert (1968), Nimmo-Smith and Raison (1968), Bruce *et al.* (1969), Smith and Brooks (1969), Zussman *et al.* (1970), Bennet and Bueding (1971, 1973), Senft *et al.* (1972, 1973a,b). Although some of the metabolic pathways and enzymic systems of schistosomes are already known, and, as it has been shown, some schistosome enzymes are not identical with those of the mammalian hosts (Mansour and Bueding, 1953; Mansour *et al.*, 1954), more fundamental data are necessary before this biochemical approach can actually serve for designing new antischistosomal drugs.

The aforementioned approaches for screening antischistosomal candi-

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dates can be performed *in vitro, in vivo* or *in vivo/in vitro*. The *in vitro* test is handicapped by the fact that metabolism of the drug may be necessary to form an active transformation product. Moreover, there is no consistent correlation between *in vitro* and *in vivo* tests. For example, diaminodiphenoxyalkanes, which are highly active against *S. mansoni in vivo* (Raison and Standen, 1955; Hill, 1956), show low activity *in vitro*, whereas alkylenebisbenzylamines, highly active *in vitro* (Bueding and Penedo, 1957), display little efficacy *in vivo* (Standen, 1963). Hycanthone, the hydroxymethyl analog of Miracil D, is highly active against infection in different laboratory animals (Rosi *et al.* 1965; Berberian *et al.*, 1967a,b) but inactive *in vitro* (Archer and Yarinsky, 1972). Lack of correlation is also observed with glucosamine and naphthoquinones (Bueding *et al.*, 1947, 1954; Brener, 1960; Pellegrino *et al.*, 1962; Standen, 1963).

Nevertheless, *S. mansoni in vitro* cultures are very useful for investigating the mechanism of action of active compounds and, afterward, for selecting the best compounds of a series through *in vivo/in vitro* tests; finally, they can also be used as a biological measure of drug concentration in body fluids.

The *in vivo* tests, although the most useful and widely employed, present some disadvantages: they are time consuming, more expensive, and require previous experiments on several animal species and different types of assessment of drug activity. For routine *in vivo* screening, the common working model in use is the white mouse, as the host, and *S. mansoni*, as the infecting agent. However, Okpala (1959) and Berberian and Freele (1964) prefer the hamster as a primary host, and Petranyi (1969) and Lämmler and Petranyi (1971) use *Mastomys natalensis* (multimammate rat).

It must be pointed out that some schistosomicidal compounds have proved highly effective in mice, but showed no, or just moderate, activity in hamsters, monkeys, or man (Standen, 1963; Lämmler, 1964, 1968; Pellegrino and Katz, 1968; Katz and Pellegrino, 1974a).

As basic criteria in the search for new chemical leads, both suppressive and curative effects have been used. Although the former seem more sensitive, as measured by the oogram method (Pellegrino *et al.*, 1962; Pellegrino and Faria, 1965), the latter are, nevertheless, generally preferred (Standen, 1967). However, any drug found to interfere with egg laying (suppressive effect) will also be evaluated as a curative agent.

During the trials for antischistosomal drugs, the degree of maturity of the infection is very important. For example, thioxanthones, antimonials, *p*-aminophenoxyalkanes, mirasans, niridazole, and other compounds show different activity when administered to animals at different times,

on the day of infection, 2–3 weeks later, or when the schistosomes are sexually mature (Kikuth and Gönnert, 1948; Watson *et al.*, 1948; Schubert, 1948c; Standen, 1955; Lämmler, 1958; Bruce *et al.*, 1962; Stohler and Frey, 1963, 1964a; Sadun *et al.*, 1966).

II. Laboratory Maintenance of *Schistosoma mansoni*

Culture and infection of *Biomphalaria glabrata* snails, as well as infection of mice, hamsters, and monkeys will now be discussed as the main steps for the maintenance of *S. mansoni*, emphasizing in particular the routine methods employed in our laboratories for screening purposes and preclinical trials in schistosomiasis.

A. Culture of Snail Vector

Biomphalaria glabrata is the most suitable snail for mass culture and for infection. It must be pointed out, however, that, as is true for several parasitic diseases, there will exist differences in the infectivity of various parasite strains and the susceptibility of the intermediate host species used. It has been clearly demonstrated that a snail serving as an intermediate host for a schistosome in one geographical area may be poorly susceptible or even refractory to infection with the same parasite from a different area (Vogel, 1942; Cram *et al.*, 1947; Files and Cram, 1949; Files, 1951; Newton, 1953; Paraense and Corrêa, 1963; Kagan and Geiger, 1965; Saoud, 1965).

As claimed by Kagan and Geiger (1965), the genetic constitution of the miracidium may be an important factor in determining its invasiveness. As a practical conclusion, for the maintenance of the parasite life cycle in the laboratory, snails and miracidia from the same endemic area are preferable.

Biomphalaria glabrata readily deposit their egg masses on shoots of *Ludwigia palustris* (Standen, 1951a) or on polyethylene plastic sheets (Olivier *et al.*, 1962) floating on the water surface. Snails kept in polyethylene bags, lay the egg masses on the inner surface of the bags (Pellegrino and Gonçalves, 1965).

In our laboratory, egg masses are obtained by floating pieces of polystyrene foam of about 10 × 15 cm in the aquaria. The snails prefer these to glass or vegetation. This method also makes handling very easy, since the pieces may be transferred to polyethylene containers, where the snails emerge. Newly hatched snails feed on the algal film that usually grows on the polystyrene foam or plastic sheets. A special food for fish is also dusted onto the water surface. After reaching the size of

about 0.5 cm, the snails are transferred to an aquarium filled with dechlorinated and artificially aerated water. The bottom of each aquarium is covered with a layer of sand and sterilized earth.

Dechlorinated water from a cement-asbestos reservoir is distributed into the aquaria at the rate of about 10 liters/day per aquarium. Excess water is automatically drained through an overflow tube. Artificial aeration and illumination are provided for 8 hours a day. Each aquarium contains 30 liters of water which is adequate for about 200 snails. The snail room is maintained at 25–27°C. Fresh lettuce and commercial fish food are used for feeding the snails. Every 2 months, the snails are removed so that the aquarium may be cleaned.

Daphnia can be used for control of microflora and fauna (Berberian and Freele, 1964), whereas snail feces and decaying vegetable or food materials are removed by the oligochete *Tubifex* (Standen, 1963).

Optimal physical conditions for *B. glabrata* culture are pH 7.2–7.8; high oxygen tension; absence of chlorine, copper and, zinc from the water; calcium carbonate concentration of approximately 18 ppm; sodium–calcium ratio of 1:1; and temperature of 23°–28°C (Cowper, 1946; Michelson, 1961; Frank, 1963; Standen, 1963). It is very important to maintain low population density and avoid crowding if good survival and reproduction are to be obtained (Chernin and Michelson, 1957a,b).

Under optimal conditions, allowing 8–9 days incubation of the snail eggs, the egg-to-egg cycle can be completed in 1 month. The reproductive potential of *B. glabrata* is about 14,000 eggs (Ritchie *et al.*, 1963, 1966a). A system for mass-producing *B. glabrata* has been described by Rowan (1958) and Sandt *et al.* (1965).

Although lettuce is generally used for breeding the snails, other types of food and formulations have been proposed, such as calcium alginate (Standen, 1951a), further modified by Moore *et al.* (1953), and a diet consisting of a mixture of Cerophyl, wheat germ cereal, Glandex fish food, and powdered milk in a ratio of 4:2:2:1, respectively (Moore *et al.*, 1953; Etges and Ritchie, 1966).

B. Infection of Snails

For the infection of *B. glabrata*, miracidia are easily obtained from eggs excreted with the feces, or retained in tissues (liver and intestine preferably) of mice and hamsters experimentally infected with *S. mansoni*. Three main methods are employed for separating the eggs: digestion, flotation, and sedimentation.

The digestion method was proposed for the isolation of schistosome

eggs from tissues of infected animals (Bénex, 1960; Smithers, 1960; Browne and Thomas, 1963). According to Smithers (1960), trypsin digestion by itself fails to remove collagen fibers. Further treatment with pepsin is necessary to digest the collagen, leaving the eggs free from the host's tissues. A final washing by centrifugation can be utilized to remove most of the dead eggs in the supernatant.

Ritchie and Berrios-Duran (1961) developed an interesting method for recovering *S. mansoni* eggs from the liver and intestine of mice and hamsters or from their feces. This technique consists of introducing 2.0% saline through a porous stone at the bottom of a sidearm Erlenmeyer flask containing the suspension of eggs and tissue in 1.7% saline. The flow (70–100 ml/minute) is continued until the suspension in 1.7% saline is entirely displaced through the sidearm overflow. The eggs fall through the interface and collect at the bottom of the flask.

In our laboratories, Standen's method (1949, 1953) is used with slight modifications. Infected mice (or hamsters) are sacrificed, the gut removed, and its superficial layer scraped into a Waring Blendor; the liver is then added, and the whole is transformed into a paste with 0.9% saline. After passing through a wide-mesh stainless steel screen more saline is added to the paste, which is allowed to sediment in the dark at 4°C. Resuspension and decanting are repeated until a clear supernatant is obtained. The final sediment is suspended in water at 28° to 30°C and placed under a bright light. The eggs start hatching within 0.5 to 1 hour. During this procedure it is important to bear in mind that salinity, low temperature, and darkness represent inhibitory conditions, whereas fresh water, higher temperature, and illumination stimulate the hatching process (Maldonado and Acosta Matienzo, 1948; Maldonado *et al.*, 1950a,b; Standen, 1951b). The average life span of the free-living miracidium is from 5 to 6 hours. About 91% of the miracidia remain active 1 hour after hatching, but only 25% after 8 hours (Maldonado and Acosta Matienzo, 1948).

Techniques for miracidium concentration based on their positive phototropism and negative geotropism have been described (Stunkard, 1946; Chaia, 1956).

Individual and mass exposure of snails to miracidia are two methods used for *B. glabrata* infection. In the former, the snails are exposed to 10 miracidia each, in small glass containers with the minimum volume of water required for snail movement (Cram, 1947; Standen, 1952). In the latter, batches of 300 *B. glabrata* in 30-liter glass aquaria are exposed to about 5000 miracidia at a temperature of 27°C. High mortality of infected snails is observed 6–7 weeks after infection, coincidental with

the emergence of large numbers of cercariae. The infected snails must be maintained at 26° to 28°C, since snails can lose their infection if they are kept at lower temperatures.

In experiments with the Belo Horizonte strain of *B. glabrata* and L. E. strain of miracidium (isolated by Pellegrino, 15 years ago in Belo Horizonte from an adult patient only once exposed to *S. mansoni* cercariae) and using the individual method of 10 miracidia for each snail, between 80–90% of the molluscs became infected, shedding large amounts of cercariae for several months.

For obtaining cercariae, 30–50 laboratory-infected *B. glabrata* are placed in a beaker with 150 ml dechlorinated water and left under an electric lamp for 2 to 3 hours. After removing the snails, the cercarial suspension is screened to retain snail feces and three 1-ml samples are counted after killing the cercariae with formalin. When only a small number of snails is used to obtain this cercarial suspension, the parasites have an unbalanced sex ratio.

C. Infection of Laboratory Animals

1. *Mouse*

The white mouse, widely used in most laboratories for screening and preclinical trials, may be easily infected by *S. mansoni* cercariae by the intraperitoneal, subcutaneous, and percutaneous routes.

Although the intraperitoneal route has been used for routine chemotherapeutic work, it must be remembered that this is an unnatural way to infect and that part of the schistosomes will not migrate to the portal system, but will remain in the peritoneal cavity (Moore and Meleney, 1955), thus hindering the assessment of therapeutic drugs.

Two methods are used for the percutaneous route: (*a*) the wading method and (*b*) the tail-immersion method.

The wading method allows the cercariae to penetrate the animal's body. After letting the mice run about in a container with warm water to stimulate voidance of feces and urine, the animals are transferred to suitable-sized wide-mouthed glass jars provided with bung and ventilating shaft. The cercarial suspension is introduced through the vent by a spring-loaded syringe. After 20 minutes the mice are removed and dried off in warm wood-wool (Standen, 1949).

The tail-immersion method consists of immersing the tails of mice into tubes containing cercariae. Previously anesthetized mice, are exposed for 45 minutes, after which the mice are returned to their cages (Olivier and Stirewalt, 1952; Stirewalt and Bronson, 1955; Pellegrino and Katz,

1968). A small plastic cage was designed by Radke *et al.* (1971) making anesthesia unnecessary.

In our laboratories, as in Hoffman-La Roche and Ciba laboratories, the subcutaneous route of infection is preferred. The cercariae (100–120 per mouse) are inoculated with a syringe into the subcutaneous tissue of the animal's back. The mean percentage of adult schistosomes recovered has been about 20% with practically all infected mice.

For exposing mice to large amounts of cercariae, the subcutaneous route should be avoided because of the large volume of suspension to be injected. In this case, the percutaneous route, already described, is preferable, using the technique of Radke *et al.* (1971).

2. *Hamster*

By the intraperitoneal route, the worm yield has been inferior to 20% (Cram and Figgat, 1947; Moore *et al.*, 1949; Berberian and Freele, 1964), no worms having been found in the peritoneal cavity after 28 days of infection (Moore and Meleney, 1955). Percutaneous exposure through the shaved skin of hamsters induces a regular infection, the recovery of worms being about 30 to 40% (Cram and Figgat, 1947; Moore *et al.*, 1949; Faria and Pellegrino, 1963).

In our laboratories, cheek pouch infection is routinely employed (Pellegrino *et al.*, 1965a). The percentage of recovery has been 30–50% adult *S. mansoni* worms.

3. *Monkeys*

Rhesus monkeys (*Macaca mulatta*) have been commonly used in schistosomiasis mansoni preclinical trials (Kikuth and Gönnert, 1948; Hill, 1956, Bruce and Sadun, 1963, 1966; Thompson *et al.*, 1962, 1965; Campbell and Cuckler, 1963; Sadun *et al.*, 1966). The use of rhesus monkeys, however, is handicapped by their cost, by difficulty in handling, and by the self-limiting nature of the infection (Vogel 1949, 1958; Lichtenberg and Ritchie, 1961; Smithers and Terry, 1965; Ritchie *et al.*, 1966b; Foster and Broomfield, 1971).

Other genera of monkeys have also been used, namely, *Cercopithecus, Papio,* and *Cebus* (Oesterlin, 1934; Newsome, 1953, 1963; Luttermoser *et al.*, 1960; Meisenhelder and Thompson, 1963; Pellegrino *et al.*, 1966, 1967a,b; Katz *et al.*, 1967b; Pellegrino and Katz, 1969, 1972; Foster, 1973; Katz and Pellegrino 1974b,c).

In our laboratories, *Cebus apella macrocephalus,* a very good host for *S. mansoni* (Coelho and Magalhães Filho, 1953; Brener and Alvarenga,

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