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Uptake of the Nitroimidazole Drug Megazol by African Trypanosomes

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ABSTRACT. Megazol, CL 64,855 (2-amino-5-[1-methyl-5-nitro-2-imidazolyl]-1,3,4-thiazole) has been shown to be extremely effective in clearing experimental infections of African trypanosomes. An unusual amino-purine transporter termed P2, implicated in the transport of both the diamidine and melaminophenyl arsenical classes of drug in *Trypanosoma brucei*, recognised chemical groups on compounds which are also present on megazol. Megazol interacted with this carrier protein, as judged by its ability to inhibit P2 adenosine transport and to abrogate *in vitro* arsenical-induced lysis in a dose-dependent manner. However, parasites resistant to melaminophenyl arsenical and diamidine drugs due to lack of the P2 transporter showed no resistance to megazol. This is because passive diffusion represented the major route of entry. Initial rates of uptake were not saturable within the limit of megazol's solubility and did not conform to thermodynamic precepts compatible with carrier-mediated uptake. Adenosine and other P2 transporter substrates, even at high concentration, had little impact on megazol uptake. Uptake was biphasic, with a very rapid equilibration across the membrane followed by a slower accumulation over time. The equilibration phase represented a simple passive diffusion, with the subsequent uptake probably being due to metabolism of the drug. BIOCHEM PHARMACOL **59**;6:615–620, 2000.

KEY WORDS. trypanosomiasis; sleeping sickness; chemotherapy; megazol; drug uptake

Human African trypanosomiasis or sleeping sickness has recently re-emerged and is prevalent in much of sub-Saharan Africa [1]. Recent reports from the WHO¶ suggest that cases are proving refractory to treatment with melarsoprol (MelB), the only drug widely used to kill the parasites once they enter the central nervous system. The breakdown of therapeutic intervention via melarsoprol is particularly worrying, since the only other drug licensed for advanced stage sleeping sickness is D,L-α-difluoromethylornithine (DFMO) [2], the long-term availability of which is uncertain. The need for new drugs for use in sleeping sickness is urgent. One compound that has been shown to have activity against both African and South American trypanosomes is megazol. This is a nitroheterocyclic compound originally described in 1968 [3] and proven to be effective against a wide range of parasitic protozoa [4]. Difficulties may surround the suitability of the drug for use in humans since it is positive in Ames tests [5]. However, since the Ames test employs *Salmonella* species with unusual nitroreductase activities [6], results from the classical test should be treated with caution in assessing nitro-containing drugs. Neither rodent [7] nor primate [8] studies have revealed appreciable toxicity in short-term testing, but possible mutagenic capability cannot be ignored and more extensive tests are required. Notwithstanding these potential difficulties, the drug has prolific activity against trypanosomiasis and a suramin–megazol combination clears the parasites from the central nervous system in a mouse model of chronic sleeping sickness [9], and megazol applied with melarsoprol will even cure the disease if applied topically [10].

Currently little is known on the mode of action of megazol. Initial reports that bioreduction had limited association in its action against *Trypanosoma cruzi* [11] have been challenged, since the drug has been shown to be subject to single electron reduction by a variety of enzymatic processes with reductase activity [12, 13], including *T. cruzi* extracts [13]. Nevertheless, the reduction potential of the compound (−438 mV) [12] is substantially lower than for nifurtimox (−260 mV) [12]. In fact, its potential is closer to that of metronidazole (−485 mV) [13], a drug with

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¶ *Abbreviations:* megazol, CL 64,855 (2-amino-5-[1-methyl-5-nitro-2-imidazolyl]-1,3,4-thiazole); DEAE-cellulose, diethyaminoethyl cellulose; IC_{50} The concentration required to inhibit 50% of uptake activity; and WHO, World Health Organisation.

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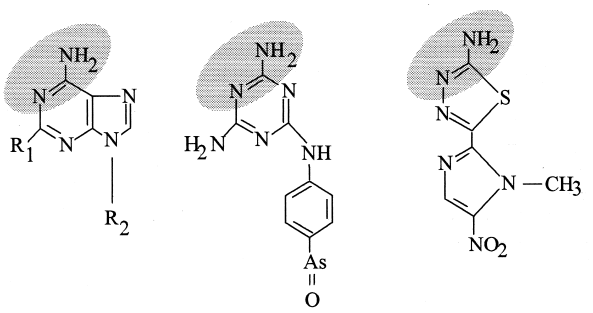

FIG. 1. Structural similarity between megazol, amino-purines, and melarsen oxide.

proven long-term safety in humans treated for disease caused by anaerobic or microaerophilic pathogens [14].

Regardless of the intracellular effects of any pharmaceutical reagent, activity often relies upon the ability of the drug to enter cells. For *Trypanosoma brucei*, an extracellular parasite, a drug that can cross the parasite's membrane but not that of host cells can be selective regardless of its intracellular targets. The melaminophenyl arsenicals and diamidines rely upon an unusual amino-purine transporter, termed P2, for uptake [15]. Loss of this transporter mediates resistance to these drugs [16]. The P2 transporter recognizes a motif common to the melamine-based arsenicals, diamidines and amino purines, which allows them to share this route of uptake [16]. Inspection of the structure of megazol (Fig. 1) shows that it shares this motif, thus suggesting that megazol may also use the same route of entry as these other drugs. Should this be the case, it would be anticipated that parasites cross-resistant to arsenicals and diamidines through loss of P2 transport function would also be resistant to megazol. This would seriously hinder its use as a potential new trypanocide, and hence it is important to understand precisely how this compound enters *T. brucei*.

MATERIALS AND METHODS

Chemicals

Megazol, tritiated at position 4, was obtained using 4-iodo-megazol, as described [17]. The reaction was performed at the 30 micromole scale. Tritiated product was purified by HPLC, on a Nucleosil C18 column, eluted with a mixture consisting of 80% H_2O–20% CH_3CN (retention time 11 min). The structure was conformed by [^{3}H] nuclear magnetic resonance δ:8.4 ppm, mass spectrometry IC/NH3 e/m: 227 = 100%; 228 = 18.4%; 229 = 83.1%. The final specific activity was 12.6 Ci per mmol. [2-^{3}H]adenosine was from Amersham. All other chemicals were from Sigma and of the highest quality available.

Cultivation of Parasites

Trypanosoma brucei brucei bloodstream from trypomastigotes (Strain 427, c118 [MITat 1.5]) [18] were grown in Sprague–Dawley rats (300–500 g) and were purified from blood using DEAE–cellulose chromatography [19], then washed three times in transport buffer (TB) (25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 0.55 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5.6 mM Na_2HPO_4, 11.1 mM glucose pH 7.4).

Uptake Studies

Parasites were diluted to a concentration of 2×10^8 mL^{-1} and stored on ice in transport buffer. Prior to use, they were warmed to the appropriate temperature, and 0.1-mL aliquots (=2×10^7 cells) were added to 0.1 mL of temperature-equilibrated transport buffer, including labelled megazol, and other compounds at the concentrations stated in the text and figure legends. Uptake was terminated by centrifuging the cells through silicone oil (1-bromododecane, 98% [Aldrich]; final density 1.038 g/mL) as described [20], then freezing immediately in a dry-ice/ethanol bath. Incorporated radio-activity was counted by liquid scintillation after cutting the bottom of the tube (containing the cell pellet, but no transport buffer) into a vial, lysing the cells with 0.2 mL of 2% SDS, and then adding 3 mL of Picofluor 40 Scintillation fluid. Parasites were viewed by light microscopy to assess viability over the course of these experiments. They remained viable and highly motile throughout, and megazol had no visible effect on viability over the short time–courses of the uptake experiments.

In Vitro *Lysis Studies*

Lysis of trypanosomes by melarsen oxide was measured spectrophotometrically by monitoring the decrease in absorbance due to light scatter induced by dying parasites at 750 nm as described [15].

Megazol Activity against Arsenical-sensitive and-resistant T. brucei In Vivo

T. brucei strain 427 c118 and an arsenical-resistant derivative, clone RU15, known to lack the P2 amino-purine transporter [15], were injected intraperitoneally into groups of five male CD1 mice (18–20 g) at a concentration of 10^5 parasites mL^{-1}. Parasites became visible in blood smears from tail venepuncture the next day and drugs were administered in a single dose by intraperitoneal injection. One control group infected with each parasite was treated with just 0.1 mL DMSO (solvent for megazol), one group was treated with melarsoprol (5 mg kg^{-1}), and other groups were treated with megazol dissolved in DMSO by 2-fold serial dilutions from 50 mg kg^{-1} to 0.78 mg kg^{-1}. Animals were inspected daily and blood monitored by phase contrast microscopy after drug administration for forty days, after which non-parasitised, living mice were considered cured.

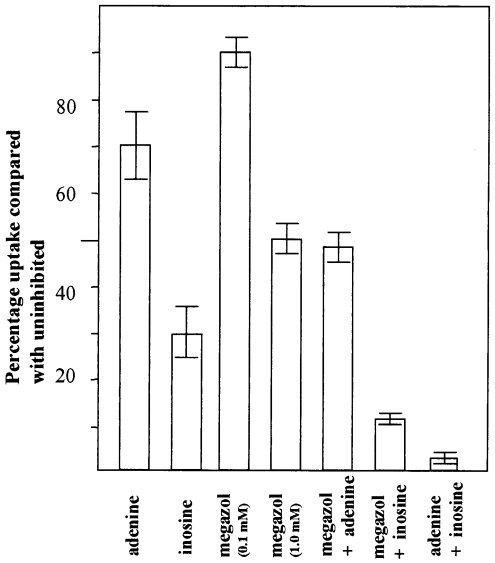

FIG. 2. Inhibition of adenosine uptake using known competitive substrates of the P1 and P2 transporters and megazol. Parasites (4×10^7) were incubated in transport buffer with 1 μM [^{3}H] adenosine with no inhibitor, 0.1 mM adenine, 1 mM inosine, or megazol (concentrations indicated), or combinations as indicated (megazol was used at 1 mM in combination with adenine and inosine). Transport was allowed to proceed for 20 sec and was terminated by centrifugation through oil. In the presence of 0.1 mM adenine, adenosine uptake was measured at 71 ± 8% of the uninhibited control; for 1 mM inosine this was 29 ± 6%; for 0.1 mM megazol 88 ± 4%; and for 1.0 mM megazol 49 ± 2%. when 1 mM megazol was combined with 0.1 mM adenine, 47 ± 2% of the uptake remained, and when 1 mM megazol was combined with 0.1 mM inosine, 12 ± 1% uptake remained Adenine (0.1 mM) combined with 1 mM inosine brought uptake to 4 ± 0.5% of the uninhibited level. Values are means ±SD.

RESULTS

Megazol Interaction with the P2 Amino-purine Transporter in T. brucei

Inspection of the structure of megazol reveals it to possess a motif common to substrates of the *T. brucei* P2 amino-purine transporter, e.g. adenosine and melarsen oxide (Fig. 1). To test whether the presence of the putative P2 recognition motif on megazol allowed this compound to interact with the transporter, the uptake of adenosine via P2 in *T. brucei* was measured in the presence of varying concentrations of megazol. The P2 transporter is responsible for approximately 30% of total adenosine uptake in *T. brucei* S427, and P1 for 70% [15]. Adenosine transport via P2 was distinguished from that of a second adenosine transporter, P1, by saturating this latter carrier with 1 mM inosine [15]. Adenosine transport via P1 was also measured by blocking P2 with 0.1 mM adenine [15]. Megazol inhibited adenosine transport via the P2 transporter in a dose-dependent manner, indicating an interaction between megazol and P2 (Fig. 2). An IC_{50} of 22.1 ± 1.6 μM was measured for megazol inhibition of adenosine uptake via the P2 transporter, indicating that the affinity of this transporter for the drug is high, albeit lower than for its physiological substrates adenosine and adenine (for which K_m values in the submicromolar range are measured [15, 16]). Uptake of melarsen oxide is also believed to occur via the P2 transporter [15]. Addition of 1 μM melarsen oxide to parasites *in vitro* caused a rapid lysis of cells as measured spectrophotometrically. Megazol inhibited this in a dose-dependent fashion, typical of other substrates for the P2 nucleoside transporter (Fig. 3), which further suggests an interaction between megazol and the transporter.

Lack of Megazol Resistance in P2 Transporter-deficient Parasites

To further assess the role of the P2 transporter in the uptake and activity of megazol, parasites lacking detectable P2 transport activity were tested for sensitivity to this drug. A *T. brucei* clone which had lost P2 nucleoside transporter activity during the derivation of melarsen resistance was tested for sensitivity to megazol and compared with wild-type cells. The concentration of megazol required to cure mice infected with arsenical-resistant cells was half that needed to cure mice infected with wild-type cells, implying an increased sensitivity in the former line. ED_{50} values were determined using a non-linear regression fit to plots of the fraction of mice affected as a function of the log of the dose of drug in mg per kg, using the Grafit software (data not shown). ED_{50} values of 3.3 mg kg^{-1} for RU15 and 8.8 mg kg^{-1} for S427 were thus calculated, revealing RU15 to be 2- to 3-fold more sensitive to megazol than is S427. The resistant line, in contrast, has over 60-fold less sensitivity to

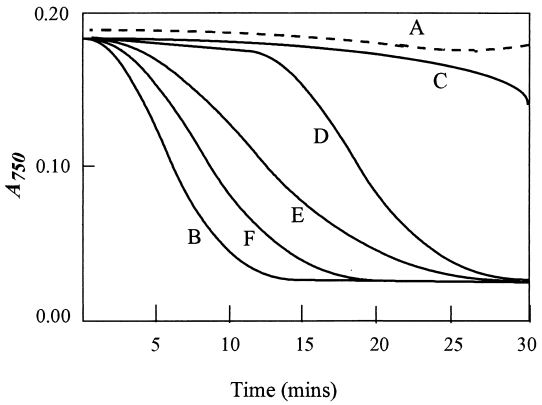

FIG. 3. Abrogation of arsenical-induced lysis by megazol. Parasites (10^7 in 10 μL) were added to transport buffer at 37° in a 1-mL cuvette with (A) 10 μL DMSO, (B) 10 μL DMSO + 1 μM melarsen oxide, (C) 1 μM melarsen oxide + 1 mM megazol, (D) 1 μM melarsen oxide + 0.2 mM megazol, (E) 1 μM melarsen oxide + 0.1 mM megazol, (F) 1 μM melarsen oxide + 0.02 mM megazol. Megazol was dissolved in DMSO to concentrations allowing to 10-μL addition to yield the final concentration. Absorbance was followed for 30 min in a thermostat-controlled Beckman DU-70 fitted with a 6-unit sample changer. As parasites lyse, light scatter is reduced, which is recorded as a decrease in absorbance by the spectrophotometer. A_{750} is the absorbance measured at 750 nm in a 1-mL cuvette containing a suspension of trypanosomes.

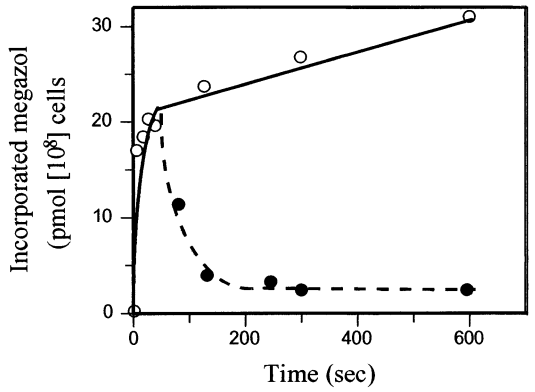

FIG. 4. Uptake and efflux of megazol by *T. brucei* as a function of time. Aliquots of 4 × 10^7 parasites were incubated in transport buffer (TB) with 10 μM megazol over a 10-min period in 100 μL of TB. Uptake was terminated at specified time points by centrifugation through oil. The dotted line represents radiolabelled megazol retained by cells after they were transferred to megazol-free buffer.

melarsoprol compared with the sensitive strain [21]. This result indicates that in spite of an ability to interact with the P2 transporter, the major route of uptake of megazol into trypansomes is independent of this carrier protein.

Megazol Uptake as a Function of Time

While megazol is clearly able to interact with the P2 transporter as judged by its ability to inhibit uptake of both adenosine and melarsen oxide, this evidence is insufficient to indicate a direct role for the transporter in uptake of the drug. Since parasites lacking P2 transport activity for adenosine remain sensitive to the drug, an alternative route of uptake seemed likely. Therefore, [^{3}H] megazol (10 μM) was added to a suspension of wild-type S427 parasites and uptake permitted to proceed for 15 min. Aliquots were isolated and accumulated label counted at different time points during the uptake period (Fig. 4). Within 30 sec, the internal concentration of drug equilibrated with that outside, assuming an intracellular aqueous volume of 1.7 μL per 10^8 cells [22]. Beyond this point, uptake entered a second, slower phase of 1 pmol [10^8 cells]$^{-1}$ min^{-1}. Such a result would be expected if internalised drug were either metabolised or sequestered in an intracellular compartment after uptake, removing internal drug from the equilibrium and allowing further entry down the resulting concentration gradient. Parasites which had been loaded with tritiated megazol were then moved to a solution without megazol and the rate of efflux from the cell measured (Fig. 4). Efflux was also very rapid, with over 90% of the label lost from the cells within 1 min. In spite of repeated washings into megazol-free buffer, a small amount of label did remain present in the cells. Transferring labelled megazol-loaded cells to buffer containing 10 μM cold megazol revealed that label left the parasites at a rate indistinguishable from that when they were added to megazol-free medium. This indicates that the internalised label is free to equilibrate with unlabelled drug and that a *trans*-acceleration process, typical of membrane transport phenomena, is not in operation.

Megazol Uptake as a Function of Substrate Concentration

Megazol is a highly amphipathic molecule and is only sparingly soluble in water. At concentrations beyond 1 mM, it precipitates out of solution. Consequently, it was possible to measure uptake as a function of substrate concentration only within a range between 1 μM and 1 mM. The rate of uptake increased linearly with megazol concentration within this range (data not shown). The process is not saturable within the range tested. It cannot be ruled out that a transporter with a K_m beyond 1 mM exists for megazol, although this could not be directly tested due to the limit of megazols' solubility.

Megazol Uptake as a Function of Temperature

Thermodynamic considerations bring most carrier-mediated uptake processes to negligible levels on ice. When the rate of uptake of 1 μM megazol into *T. brucei* over 60 sec was compared at 0° and 25°, no significant difference was apparent (Fig. 5).

Lack of Inhibition of Megazol Uptake by Nucleosides

Megazol does interact with the P2 transporter and inhibits uptake of adenosine and melarsen oxide by this route. The major route of uptake, however, appears to be via passive diffusion. The extent to which interaction with the P2 transporter makes a contribution to net uptake was determined by testing the ability of high concentrations of various purines and pyrimidines to abrogate uptake of 1 μM megazol over a 10-sec period. The P2-specific substrates

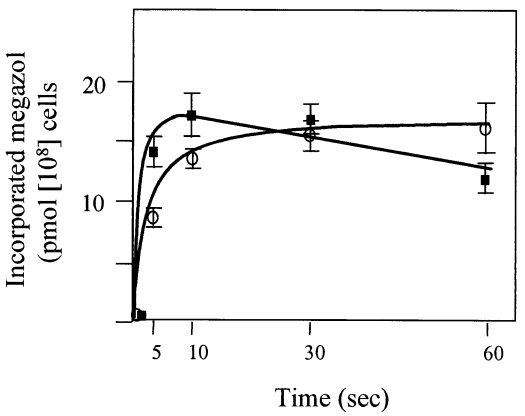

FIG. 5. Uptake of megazol by *T. brucei* as a function of temperature. Aliquots of 4 × 10^7 parasites were incubated in transport buffer with 10 μM megazol, and aliquots were sampled for uptake at set time points over a 1-min period. Uptake was allowed to proceed at room temperature (filled squares) and on ice (open circles). Values are means ±SD.

adenine and adenosine, when used at 0.1 mM and 1 mM (their respective saturating concentrations [15]), inhibited less than 10% of uptake, and other nucleosides (inosine and thymidine at 1 mM) had no inhibitory effect on megazol uptake (data not shown). These results indicate that while megazol can interact with the P2 transporter, its uptake is largely independent of this carrier protein.

DISCUSSION

The resurgence of sleeping sickness in Africa [1] combined with an increase in resistance to melarsoprol used in the therapy of this disease means that the development of new drugs for use against the disease is of profound importance. The nitroimidazole compound, megazol, has been shown to have activity against both African [7] and South American [4] trypanosomes. Against *T. brucei*, the compound has been shown to be active following single-dose administration in both rodent and primate models. Moreover, when administered along with suramin, the drug has been shown to be effective even when parasites have become manifest in the central nervous system [9]. It has considerable potential as a new clinically relevant trypanocide.

Megazol bears the chemical motif resembling that recognised by the P2 amino-purine transporter [16]. This carrier protein also accumulates the melaminophenyl-based arsenicals [15] and diamidines [23, 16] in trypanosomes. Its loss mediates cross-resistance between these two classes of drug [16]. If megazol were also dependent upon this carrier protein for its uptake into trypanosomes, it would not be useful against cases of sleeping sickness resistant to arsenicals due to altered P2 activity. Results presented here show that megazol can indeed interact with the P2 transporter, since it inhibits adenosine uptake via this transporter with an IC_{50} of 22 μM, indicating a high affinity, albeit over one order of magnitude lower than the affinity for natural amino-purine substrates of the transporter. Megazol also abrogates melarsen oxide-induced cell lysis in a spectrophotometric assay. However, arsenical-resistant parasites which have lost the P2 transporter remain sensitive to megazol. This can be explained by the fact that in spite of its ability to interact with the P2 transporter, the molecule appears to be accumulated directly across the membrane by passive diffusion. Evidence that passive diffusion drives megazol's uptake into *T. brucei* comes from several different experiments. Within the limits of megazol's solubility, the rate of uptake had a linear relationship with concentration, implying that the process was not saturable. If megazol were transported by a protein which recognised this compound with low affinity (K_m exceeding its solubility limit), a similar result would be expected. However, uptake of labelled megazol does not conform to the standard thermodynamic constraints imposed upon protein carriers, i.e. the rate the uptake is very similar at 25° and on ice. This indicates that megazol traverses the plasma membrane predominantly by passive diffusion. Uptake of other nitroimidazoles follows the same route in trypanosomatids [24] and other protozoa [25].

Uptake of the drug as a function of time revealed two distinct phases: an initial rapid accumulation of drug in which the concentration inside the cell equilibrated with that outside followed by a slower accumulation. Such an increase could be accounted for if the drug were leaving free solution within the cell, thus maintaining a gradient down which further drug would enter. Sequestration of the drug within an intracellular compartment could mediate such a process. A second possibility is that the drug is metabolised within the cell, thus maintaining a gradient for the non-reduced prodrug. Nitroheterocyclic compounds are generally believed to act once the nitro group has been reduced, producing reactive nitrogen species which damage cellular macromolecules directly or through the production of reactive oxygen species [26]. Recently, it has been revealed that *T. cruzi* contains a megazol reductase activity [13].

Since megazol enters parasites via passive diffusion, in spite of its ability to interact with the P2 transporter, melarsen-resistant lines should remain sensitive to this drug. In fact, parasites lacking the P2 transporter are 2- to 3-fold more sensitive to megazol than wild-type. Hypersensitivity to another nitroheterocyclic compound, tryparsamide, in parasites resistant to a non-melamine-based arsenical was previously reported [27]. A general correlation between hypersensitivity to nitroheterocyclics in arsenical-resistant parasites can be ruled out, however, as the arsenical-resistant line used here, RU15, and its wild-type parent, S427, show identical sensitivity to nifurtimox [21]. It should also be noted that the RU15 line also possesses other biochemical alterations, including changes in intracellular thiols [21], which could contribute to altered sensitivity to other compounds.

The present study reveals that megazol is capable of interacting with the exofacial binding site of the P2 amino-purine transporter. However, uptake is mediated predominantly via passive diffusion, signifying that cells resistant to melaminophenyl arsenicals and diamidines due to loss of the P2 transporter are not cross-resistant to megazol. This indicates that should the drug pass tests that lead to its gaining a license, it would be useful even against parasites resistant to these other classes of drug.

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