BIOCHEMICAL PHARMACOLOGY 75 (2008) 1580–1587

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Accumulation of the antimalarial microtubule inhibitors trifluralin and vinblastine by *Plasmodium falciparum*

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ARTICLE INFO

Article history:
Received 8 November 2007
Accepted 4 January 2008

Keywords:
Malaria
Trifluralin
Vinblastine
Accumulation
Microtubules

ABSTRACT

Malaria is a disease in desperate need of new chemotherapeutic approaches. Certain microtubule inhibitors, including vinblastine and taxol, have highly potent activity against malarial parasites and disrupt the normal microtubular structures of intra-erythrocytic parasites at relevant concentrations. While these inhibitors are useful tools, their potential as anti-malarial drugs is limited by their high toxicity to mammalian cells. In contrast, two classes of antimitotic herbicide, namely dinitroanilines (e.g. trifluralin and oryzalin) and phosphorothioamidates (e.g. amiprophosmethyl), exhibit moderate activity against the major human malarial parasite *Plasmodium falciparum* in culture but very low mammalian cytotoxicity. We examined the dynamics and kinetics of uptake and subcellular compartmentation of [^{14}C]trifluralin in comparison with [^{3}H]vinblastine. We wished to determine whether the relatively modest activity of trifluralin was the consequence of poor uptake into parasite cells. Trifluralin accumulated in parasite-infected erythrocytes to ~300 times the external concentration and vinblastine at up to ~110 times. Accumulation into uninfected erythrocytes was much lower. Uptake of trifluralin was rapid, non-saturable and readily reversed. It appears that the hydrophobic nature of trifluralin leads to accumulation largely in the membranes of the parasite, reducing the levels in the soluble fraction and limiting access to its microtubular target. By contrast, vinblastine accumulated predominantly in the soluble fraction and uptake was saturable and mostly irreversible, consistent with binding predominantly to tubulin. The results indicate that synthesis of more polar trifluralin derivatives may be a promising approach to designing microtubule inhibitors with more potent antimalarial activity.

1. Introduction

With the continuing high incidence of malaria and the spread of resistance to several established antimalarial drugs, there is a crucial need for new antimalarial agents with novel chemical structures and molecular targets [1,2]. One suggestion is that microtubule inhibitors may have promise as antimalarial agents, because microtubules play such important roles in cell division, motility and structural integrity of malarial parasites [3,4]. Certain microtubule inhibitors, including '*Vinca*' alkaloids, dolastatins and taxoids, have highly potent activity against malarial parasites and disrupt the normal microtubular structures of intra-erythrocytic parasites at very low concentrations [5–7]. While these inhibitors are useful tools, their potential as anti-malarial drugs is limited by their high toxicity to mammalian cells [4,5]. In contrast, two classes of

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.01.002

antimitotic herbicide, namely dinitroanilines (e.g. trifluralin and oryzalin) and phosphorothioamidates (e.g. amiprophos-methyl), exhibit moderate (low μM IC_{50}) activity against *Plasmodium falciparum* in culture [8–11] but very low or undetectable mammalian cytotoxicity [12–14]. It has also been demonstrated that [^{14}C]trifluralin interacts with recombinant α- and β-tubulin from *P. falciparum* to a far greater extent than to bovine brain tubulin [11]. Given their selective activity against parasites and affinity for parasite tubulin, these antimitotic herbicides may form the basis for development of novel antimalarial agents.

This study focuses on two antimitotic agents, the '*Vinca* alkaloid' vinblastine, isolated from the Madagascar periwinkle *Catharanthus roseus* (formerly *Vinca rosea*) and trifluralin, a synthetic dinitroaniline. While both these agents cause breakdown of mitotic microtubular structures, they differ greatly in their potency against asynchronous asexual cultures of *P. falciparum* [4,11]. Vinblastine has a reported 72-h IC_{50} against these cultures of 250 nM [5] while that for trifluralin under the same assay conditions is 2.9 μM [11]. Studies in mammalian cells have shown that among '*Vinca* alkaloids' there was a close correlation between cytotoxicity and efficiency of drug accumulation [16]. A similar correlation between cytotoxicity and efficiency of drug accumulation was noted for vinblastine and the more potent dolastatin 10, again in mammalian cells [17]. We report here the results of the first investigation into the accumulation of microtubule inhibitors into erythrocytes infected with *P. falciparum*. We wished to determine whether the relatively modest activity of trifluralin could be accounted for by limited uptake into parasite cells. Understanding of the factors limiting accumulation of trifluralin into parasites and comparison with uptake of the more potent vinblastine may aid in the design of new dinitroaniline derivatives with higher antimalarial potency.

2. Materials and methods

2.1. Reagents

[^{14}C]Trifluralin (16.8 mCi/mM) and [^{3}H]vinblastine (9.8 Ci/mM) were obtained from Sigma Aldrich, Dublin, Ireland and Amersham Biosciences, Dublin, Ireland, respectively. Unlabeled trifluralin and vinblastine were dissolved in dimethylsulphoxide and deionised H_2O, respectively. All chemicals were of cell culture grade and purchased from Sigma Aldrich, Dublin, Ireland unless otherwise stated.

2.2. Cell culture

P. falciparum strain 3D7 (obtained from M. Grainger, National Institute of Medical Research, London, UK) was cultivated in human O^+ erythrocytes as previously described [11]. Parasitaemias were determined by examination of Giemsa-stained smears. Age-selection of the parasites was carried out by two-step sorbitol treatment as described by Nankya-Kitaka et al. [18]. Parasitised and non-parasitised erythrocytes were separated either by Percoll®-alanine gradient as described [19,20] or by magnetic separation on a VarioMACS system (Miltenyi Biotec, Surrey, UK) according to manufacturer's instructions.

2.3. Measurement of accumulation of radiolabeled compounds by intact parasitized and unparasitized erythrocytes

Concentrations of both agents used in these studies were related to their respective IC_{50} values for late trophozoites/early schizonts [5,11]; for this reason there is a large difference in the concentrations used.

Measurements of cellular uptake of [^{14}C]trifluralin and [^{3}H]vinblastine were carried out using cultures of intact infected erythrocytes of final haematocrit 1–2% and >85% parasitaemia or uninfected erythrocytes at haematocrit 1–2%. Cells were suspended in the appropriate buffer containing 3 μM [^{14}C]trifluralin or 25 nM [^{3}H]vinblastine and incubated at 37 °C. Duplicate 200-μl samples were taken at graded time intervals and the reaction terminated upon centrifugation of the cells (14,000 × *g* for 2 min) through silicon oil (Dow Corning) and processed for scintillation counting as described previously [21]. Parasite-specific uptake was calculated by subtracting the accumulation into an equal number of uninfected erythrocytes. Unless otherwise stated, accumulation is expressed as the cellular accumulation ratio (CAR) which is the ratio of radiolabeled compound in the parasites to that remaining in a volume of buffer equal to the calculated volume of the cells following incubation.

To determine the approximate subcellular location of each compound, cells were "loaded" with either [^{14}C]trifluralin or [^{3}H]vinblastine under the conditions described for uptake experiments. Following 2 h incubation at 37 °C parasites were released from infected erythrocytes using 0.1% (w/v) saponin in ice-cold salt sodium citrate (SSC) as previously described [22]. Crude parasite extracts were then prepared by three cycles of freeze-thaw lysis followed by centrifugation at 14,000 × *g* for 1 h at 4 °C. Samples from both the pellet (particulate) (p) and soluble (s) fraction were taken and processed for scintillation counting. Protein concentrations of all fractions were obtained using the Bradford assay [23]. The distribution of tubulin was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by western blotting using anti-β-tubulin antibodies (affinity purified rabbit antiserum to a synthetic *P. falciparum* β-tubulin peptide: [24]).

To measure the effect of unlabeled compound on accumulation of [^{14}C]trifluralin and [^{3}H]vinblastine a series of solutions of twice the concentrations to be tested (0.1 μM [^{14}C]trifluralin and increasing concentrations of unlabeled trifluralin or 5 nM [^{3}H]vinblastine and increasing concentrations of unlabeled vinblastine) were prepared. A sample of schizont-infected erythrocytes (approximately 38–44 h post-invasion, selected by sorbitol treatment) was added to each solution to yield final haematocrits of 1–2% and parasitaemias of >90%. Samples were mixed and incubated at 37 °C for 2 h. Cellular accumulation of the radiolabelled compound was measured as before.

To determine the proportion of compound reversibly accumulated in schizont-infected erythrocytes, cells were