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

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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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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

"loaded" with either [^{14}C]trifluralin or [^{3}H]vinblastine under the conditions described for uptake experiments. Following 2 h incubation at 37 °C duplicate 200-μl samples were removed for determination of total cell-associated compound. To measure reversible accumulation cultures were washed 10 times by centrifugation (14,000 × *g*, 2 min) and resuspended in warm RPMI 1640 supplemented with 50 μg/ml sodium bicarbonate, with duplicate 200-μl samples removed at each stage, and cell-associated radioactivity determined as above.

Rates of compound release were measured by "loading" schizont-infected erythrocytes with either [^{14}C]trifluralin or [^{3}H]vinblastine under the conditions described for uptake experiments. Following 2 h incubation at 37 °C duplicate 200-μl samples were removed for determination of total cell-associated compound at time zero. Following centrifugation of the remaining culture (14,000 × *g*) and resuspension in warm growth medium (RPMI 1640 supplemented with 25 mM HEPES, 50 mg/l hypoxanthine, 0.18% (w/v) sodium bicarbonate, 10 mg/l gentamicin and 0.5% (w/v) Albumax II (Gibco, Auckland, New Zealand)), the resulting cell suspensions were incubated at 37 °C and at graded intervals 200-μl samples were removed and cell-associated radioactivity was determined as before.

2.4. *Measurement of susceptibilities of P. falciparum cultures of different parasitaemias to trifluralin treatment*

Susceptibility of asynchronous parasites to trifluralin was determined in 96-well microplates using the parasite lactate dehydrogenase (pLDH) method [25]. Starting cultures were of 0.2% and 1.5% parasitaemia and pLDH activity was determined following 48 and 72 h of incubation.

3. Results

3.1. *Extent and kinetics of accumulation and effect of developmental stage of parasite*

Both microtubule inhibitors accumulated to intracellular levels far in excess of those in the external medium, with CAR of ~300 for trifluralin and up to ~110 for vinblastine. Accumulation of both compounds in intact erythrocytes parasitized with both trophozoite- and schizont-stage parasites was shown to be rapid. Trifluralin accumulation reached an apparent maximum within 10 min (Fig. 1A), while vinblastine reached an apparent plateau following 15–20 min of exposure (Fig. 1B). In addition to the rapid accumulation, Fig. 1A also shows that accumulation of trifluralin in parasitized erythrocytes was unaffected by the stage of parasite present, with both trophozoite- and schizont-infected cultures exhibiting comparable kinetic curves and CARs. Due to the need for a parasitaemia in excess of 85% to reduce the impact of accumulation in uninfected erythrocytes, ring-infected erythrocytes were excluded from the current study as such high parasitaemias could not be achieved in these cultures. In contrast, vinblastine accumulation in schizont-infected erythrocytes was ~2-fold higher than that observed for trophozoite-infected erythrocytes (CAR ~110 vs. 55), though the kinetics appeared unaffected (Fig. 1B). The observation for vinblastine would concur with the increase in quantity of the presumed target, tubulin, in the latter stages of the intraerythrocytic cycle of the parasite [26].

Uninfected erythrocytes showed much lower accumulation of both compounds: as these cells lack microtubules this is as expected. However the CAR were still approximately

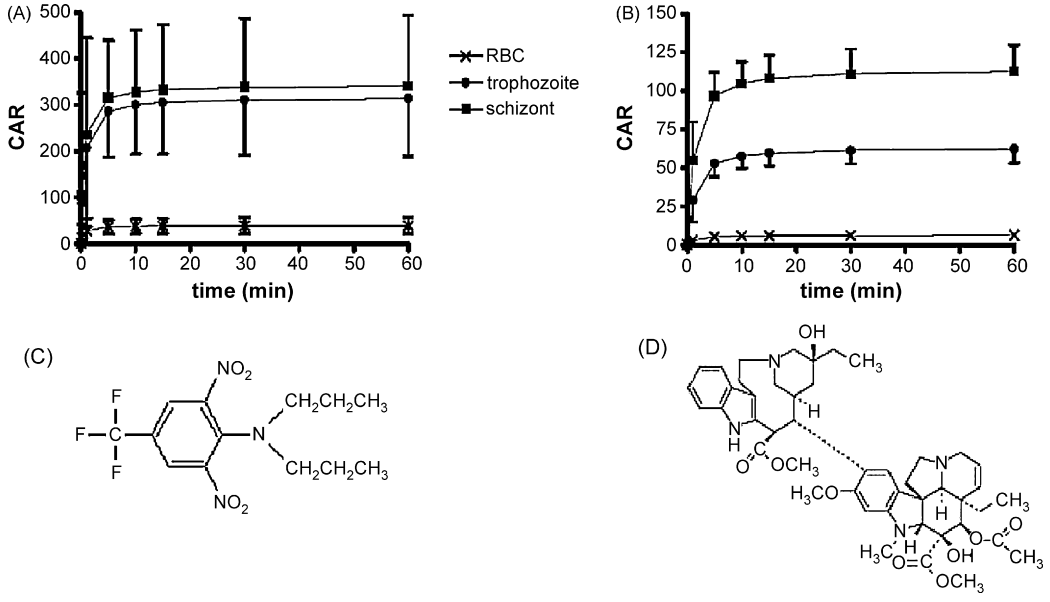

Fig. 1 – Accumulation of [^{14}C]trifluralin (A) and [^{3}H]vinblastine (B) in uninfected erythrocytes (RBC) or those infected with trophozoite- or schizont-stage *P. falciparum* parasites. Parasite cultures were incubated with labelled compound, samples were taken over a 1-h period and cell-associated accumulation of the compounds was measured by scintillation counting. Accumulation is shown as the cell accumulation ratio (CAR) and results shown represent the mean ± 95% CI for at least three replicate experiments. Error bars are shown below the data point for trophozoite-infected RBC and above it for RBC infected with schizonts. The structures of each compound are also shown (C and D).

20–40 for trifluralin and between 1 and 5 for vinblastine (Fig. 1). Previous studies with chloroquine have shown a CAR of 14 in uninfected erythrocytes [27]. There is no known erythrocytic target for any of these compounds so the differing ability of erythrocytes to concentrate them is unexplained. It is possible however that as both vinblastine and chloroquine have much higher aqueous solubilities (both defined as ''freely soluble in water'' by the Merck Index [28]) than trifluralin (0.2 to 0.4 mg/l [29]), trifluralin accumulates to a higher level in the lipophilic interiors of plasma, organellar and vesicular membranes than does vinblastine (or chloroquine).

3.2. *Subcellular locations of [^{14}C]trifluralin and [^{3}H]vinblastine*

To investigate whether both compounds co-located with their microtubular targets, a crude sub-cellular fractionation was carried out on isolated parasites dividing the cells into particulate and soluble fractions. Fig. 2A shows the presence of β-tubulin mainly in the soluble fraction, which includes the cytosol and presumably the nuclear lumen, with little in the membrane-containing particulate fraction. Although tubulins are the presumed molecular targets of trifluralin [11] the distribution of [^{14}C]trifluralin was quite different from that of β-tubulin. Instead of a higher level in the soluble fraction, trifluralin was distributed fairly evenly with a ratio of approximately 5.5:4.5 particulate: soluble (Fig. 2B). This is consistent with both binding to a cytosolic target, presumed to be microtubules, and accumulation in membranes, presumably due to the high lipophilicity of trifluralin. The distribution of [^{3}H]vinblastine more closely mimics that of β-tubulin with ~75% co-locating with tubulin in the soluble fraction (Fig. 2B).

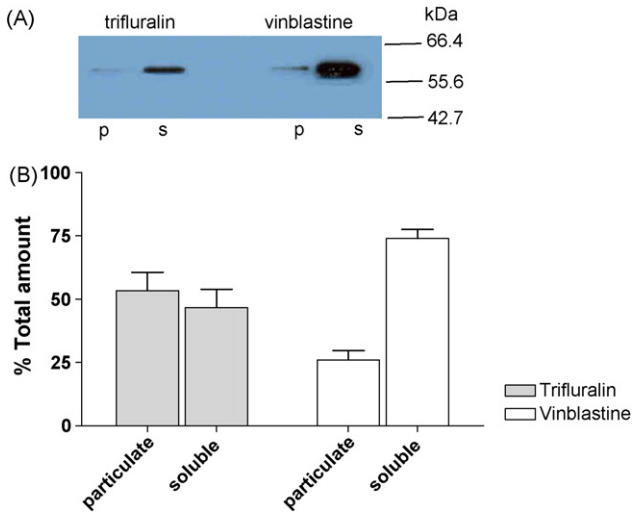

Fig. 2 – Subcellular distributions of trifluralin and vinblastine in relation to tubulin. Schizont-infected erythrocytes were incubated in the presence of either [^{14}C]trifluralin or [^{3}H]vinblastine for 2 h, freed from the erythrocytes, broken by freeze-thawing and separated by centrifugation into two fractions, particulate and soluble. Samples were analysed by SDS-PAGE followed by western blotting using anti-β-tubulin antibodies (A) and by scintillation counting (B). All lanes contain 1.5 μg total protein and the running positions of molecular-mass markers are indicated to the right of panel (A). The data in (B) represent the mean ± SEM for at least three replicate experiments. The y-axis shows the amount of compound as a percentage of the total amount.

3.3. *Effect of unlabeled trifluralin or vinblastine on the accumulation of their respective radiolabeled forms*

The effect of increasing concentrations of unlabeled compound on the ability of schizont-infected erythrocytes to accumulate the labelled form was examined. The poor solubility and low specific activity of trifluralin prevented a broader range of concentrations being used than those shown in Fig. 3A. However, it was shown that the addition of 1000-fold excess of unlabeled compound had little effect on the ability of the cells to accumulate [^{14}C]trifluralin (Fig. 3A). This indicates that at these concentrations no saturation of either target or transport mechanism (passive or active) occurs. This is not replicated for vinblastine, where a significant reduction in accumulation of the radiolabelled drug was seen as the unlabeled compound concentration was increased (Fig. 3B).

3.4. *Reversibility of accumulation of [^{14}C]trifluralin and [^{3}H]vinblastine*

Previous work with the dinitroaniline oryzalin indicated that accumulation could be reversed by repeated washing in herbicide-free medium [30]. A similar result was noted for trifluralin accumulation in *P. falciparum*-parasitized erythrocytes with only ~15% of the accumulated compound remaining after 10 washes. The reduction was continuous with additional trifluralin lost following each subsequent wash (Fig. 4A and data not shown). This would indicate that a high proportion of the compound is accumulated via a reversible low-affinity association. In contrast, ~65% of accumulated vinblastine was retained by the parasitized erythrocytes following multiple washes (Fig. 4B). The reduction in accumulated vinblastine was noted following the first and possibly the second wash with little or no significant release of vinblastine after this (Fig. 4B and data not shown). So while there does appear to be some level of low-affinity association of vinblastine, most of the drug is retained by the cells through binding to a higher-affinity target.

3.5. *Release of [^{14}C]trifluralin and [^{3}H]vinblastine by intact schizont-infected erythrocytes*

Mammalian tumour cell resistance to vinblastine has been attributed to the active efflux of the drug through a P-glycoprotein pump [31]. No such release of either vinblastine (Fig. 5B) or trifluralin (Fig. 5A) was noted following 24 h incubation of parasitized erythrocytes pre-loaded with one of the radiolabelled compounds. A reduction in accumulated compound was seen immediately following the wash step prior to re-incubation but this could be attributed to reversible or low affinity binding of the compound and not to an active efflux process.

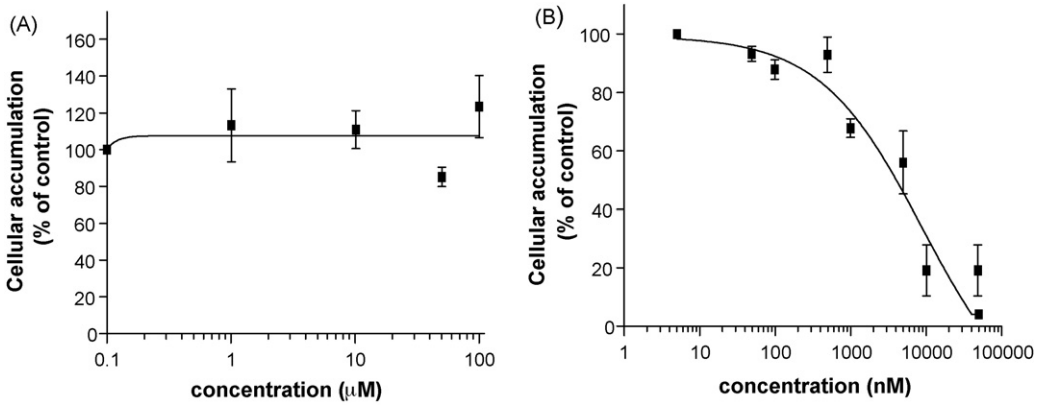

Fig. 3 – Saturability of trifluralin and vinblastine uptake. Schizont-infected erythrocytes were incubated with 0.1 μM [^{14}C]trifluralin and increasing concentrations of unlabelled trifluralin or 5 nM [^{3}H]vinblastine and increasing concentrations of unlabelled vinblastine for 2 h at 37 °C. Samples were then taken and processed for scintillation counting. The cumulative concentration of both the labelled and unlabelled compound is plotted on the *x*-axis. The *y*-axis shows the amount of cell-associated compound present after 2 h as a percentage of that found when no unlabeled compound was present. Results shown represent the mean ± SEM for at least three replicate experiments.

3.6. *Susceptibilities of asynchronous P. falciparum cultures of different parasitaemias to trifluralin treatment*

Non-specific trifluralin accumulation would reduce the amount of compound available to inhibit its tubulin target. Therefore, we expected that there would be a marked inoculum effect on susceptibility to trifluralin, such that the herbicide would be less effective on cultures of higher parasitaemia. This was shown to be the case, with 48- and 72-h IC_{50} values of ~1.3 and ~0.5 μM, respectively for cultures with an initial parasitaemia of 0.2%, compared with ~3.9 and ~1.4 μM for cultures of initial parasitaemia 1.5% (Fig. 6).

4. Discussion

A number of microtubule inhibitors have been shown to inhibit growth of erythrocytic malarial parasites [4]. Some, including the anticancer agents vinblastine, dolastatin 10 and taxol, have highly potent antimalarial activity but are equally active against mammalian cells [4–7]. Others, especially the dinitroaniline herbicides, of which trifluralin has been most investigated, are less potent but do not affect mammalian cells [5]. An obvious goal would be to design an antimalarial compound that would combine the potency of the anticancer microtubule inhibitors with the selectivity of the herbicides. In order to investigate the reasons for the modest potency of trifluralin, we asked whether uptake of the compound into erythrocytic parasites might be a problem. We assessed the uptake of labelled trifluralin alongside that of labelled vinblastine, for which we expected there would be no major barriers to penetration. It has been suggested that the hydrophobic nature of trifluralin may restrict its access to intracellular targets, thus increasing the dose required to effectively kill the target organism [32]. Previous attempts to synthesise derivatives with polar groups have resulted in compounds with greater activity against human parasites such as trypanosomes and *Cryptosporidium parvum* but there

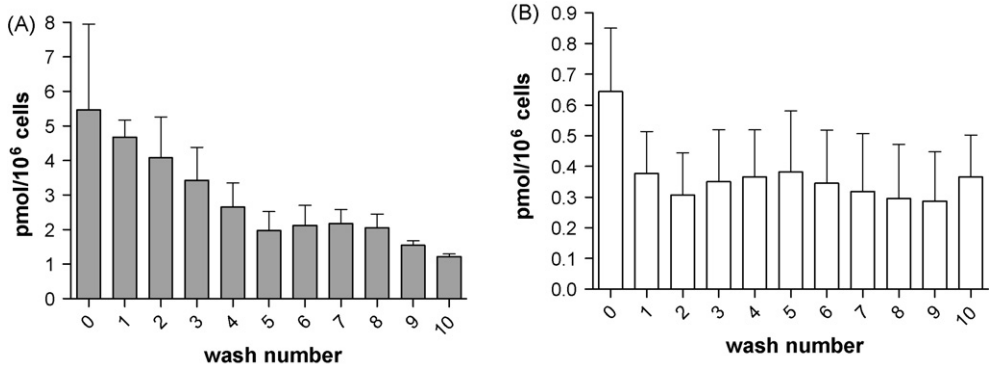

Fig. 4 – Release of [^{14}C]trifluralin (A) and [^{3}H]vinblastine (B) accumulated in schizont-infected erythrocytes following repeated washing of the cells. Cultures were loaded with radiolabelled compound as described in Section 2 then washed repeatedly by centrifugation and resuspension in medium. Samples were taken following each wash. Results represent the mean ± SEM for four replicate experiments.

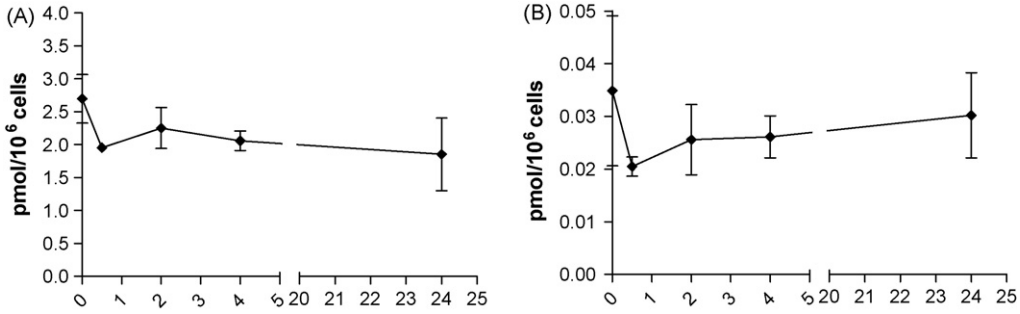

Fig. 5 – Time course of release of [^{14}C]trifluralin and [^{3}H]vinblastine accumulated in schizont-infected erythrocytes. Schizont-infected erythrocytes were incubated with either [^{14}C]trifluralin (A) or [^{3}H]vinblastine (B) for 2 h at 37 °C and cells were then washed in warm PBS twice and re-incubated in compound-free medium. Cell-associated compound was measured by scintillation counting. Results represent the mean ± SEM for three replicate experiments.

are as yet no publications to show that this works in *Plasmodium*.

Trifluralin and vinblastine were both concentrated by parasitised erythrocytes. Trifluralin achieved a CAR 3- to 6-fold higher than vinblastine in *P. falciparum* infected erythrocytes, reaching a final cell associated concentration of approximately 5.5 pmol/10^6cells compared to 0.65 pmol/10^6 cells for vinblastine. No significant differences in the kinetics of uptake were noted, with both trifluralin and vinblastine accumulating rapidly in cells. Due to the increased quantity of microtubular target in schizont-stage parasites relative to trophozoites it was expected that there would be a corresponding increase in cell-associated compound in schizonts. This was true for vinblastine, with a 2-fold higher CAR in schizont-infected erythrocytes. It was however not the case for trifluralin, where the CAR for the two stages were similar.

Unlike vinblastine, which accumulated to a modest level in uninfected erythrocytes, trifluralin appeared to be concentrated in these cells by a factor of approximately 30. When compared to other anti-malarial agents such as chloroquine, which also have no known target in erythrocytes, this concentration of trifluralin is unusually high. Based on the hydrophobic nature of the trifluralin molecule and the observation that the majority of the labelled trifluralin was found in the particulate fraction of uninfected erythrocytes (data not shown), it is possible that this is a result of association with membrane lipids. One might expect even higher accumulation in host cells with extensive internal membrane structures. This association is further indicated by the higher than expected level of association of trifluralin with the particulate fraction of the parasites, although the possibility that there is additional non-specific association with erythrocyte membrane/cytoskeletal proteins cannot be discounted based on the results shown here. Vinblastine predominantly co-located with β-tubulin in the soluble fraction of parasites, which is consistent with tubulins being the primary target of this drug, with very little associated with the particulate fraction. Accumulation in the membrane could also mask any increase in trifluralin accumulation due to an increase in the concentration of target tubulin.

Experiments on the effect of unlabeled vinblastine on accumulation of the labelled form indicated that accumulation of vinblastine was saturable. As this drug is known to bind to one site on the tubulin dimer, which represents a finite number of target sites, this effect is likely to be due to saturation of target. Uptake of trifluralin was non-saturable, consistent with a high level of non-specific uptake which may mask any saturation of the microtubular target. Further evidence for a low-affinity association of trifluralin with a cellular target was the ease with which accumulation is reversed by several wash steps. Almost 85% of cell-associated

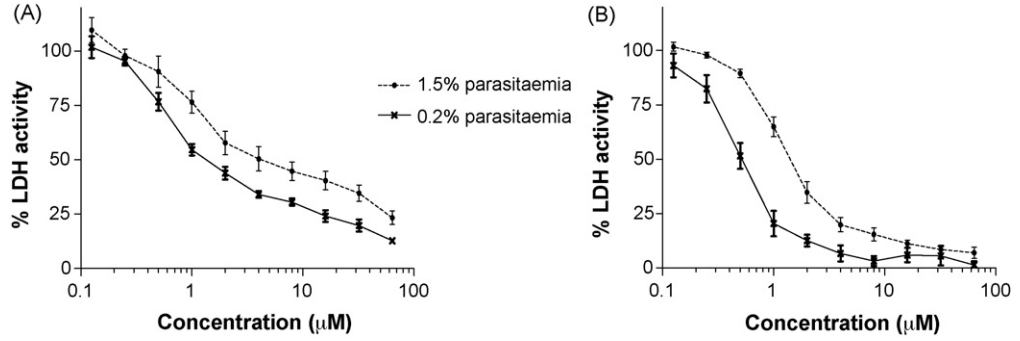

Fig. 6 – Susceptibilities of asynchronous *P. falciparum* cultures of different initial parasitaemias to trifluralin treatment. Cultures were incubated with a range of inhibitor concentrations in 96-well plates and growth assessed using the lactate dehydrogenase (LDH) method. Geometric mean values ± SEM of eight determinations after 48 h (A) and 72 h (B) are shown. Initial parasitaemias were 0.2% (crosses) or 1.5% (circles).

trifluralin was removed following ten wash steps. In contrast, approximately 65% of accumulated vinblastine was retained by the parasitized erythrocytes following multiple washes. So, while there does appear to be some level of low-affinity association of vinblastine, most of the drug is retained by the cells through binding to a higher-affinity target.

The contention that partitioning of trifluralin into membranes is reducing the amount available to bind to its target can be corroborated by the presence of an inoculum effect. Following both at 48 and 72 h incubation, cultures with an initial parasitaemia of 0.2% had an IC_{50} value ~3-fold lower that the otherwise identical cultures with initial parasitaemia of 1.5% (Fig. 6). The situation may, however, be more complex than this. The accumulation in the cyosolic fraction was ~50% of the whole and if this were solely due to binding to tubulin dimers or microtubules, some level of saturation should still be evident. As no saturation was observed, it is possible that there is non-specific binding to one or more parasite cytosolic target(s) in addition to accumulation in the membrane and that the combination of these two effects masks any saturation of the specific binding to tubulin targets.

There was no detectable efflux of either trifluralin or vinblastine. Active efflux may be a mechanism of conferring resistance to certain agents such as quinolines in *P. falciparum* [33]. The lack of evidence for the active efflux of either of these compounds may be a promising indication for the future potential of microtubule inhibitors as antimalarial drugs, but this may not hold true for derivatives of these agents.

In summary, our results indicate that accumulation of trifluralin into parasites is not a barrier to its antimalarial potency but partitioning in both erythrocyte and parasite membranes may reduce the amount of compound available to bind the target. This suggests that less lipophilic trifluralin derivatives may be more effective provided the tubulin-binding moiety is not adversely affected. Reduced lipophilicity may however incur a penalty in terms of reduced ability to cross membranes. It will therefore be important to measure the binding affinity of trifluralin for tubulin and microtubules and to investigate whether derivatives with higher affinity can be designed.

Acknowledgements

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