In search of a selective inhibitor of the induced transport of small solutes in *Plasmodium falciparum*-infected erythrocytes: effects of arylaminobenzoates

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Following invasion of the human erythrocyte by the malaria parasite, *Plasmodium falciparum*, there appear in the parasitized cell new, high-capacity permeation pathways that transport a diverse range of low-molecular-mass solutes. In this study a series of 16 arylaminobenzoates, analogues of the Cl[−] channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), were tested for their effects on the transport of choline, a univalent cation, into malaria-infected cells. A number of the arylaminobenzoates were found to be potent inhibitors of malaria-induced choline transport and to be similarly effective at blocking the induced transport of the uncharged pyrimidine nucleoside thymidine and the univalent anion lactate. The data are consistent with the hypothesis that much of the induced

transport of cations, anions and non-electrolytes into parasitized cells is via broad-specificity, anion-selective pathways of a single type. A comparison of the effects of the arylaminobenzoates on malaria-induced transport with their effects on a number of representative anion transport systems in normal mammalian cells suggests that it is possible to identify pharmacological agents that block the malaria-induced pathway while not significantly affecting important transport mechanisms in host tissues. The most potent of the induced-transport inhibitors identified were shown to inhibit [³H]hypoxanthine incorporation in *in vitro* parasite growth assays. These data support the view that the induced-transport pathway may be a viable pharmacological target.

INTRODUCTION

Human erythrocytes infected with the mature (trophozoite) form of the malaria parasite, *Plasmodium falciparum*, show increased permeability to a wide variety of small solutes including polyols, amino acids, nucleosides and organic and inorganic ions (reviewed in [1–8]). The available data are consistent with the view that much of the malaria-induced solute transport is via pathways of a single type, which take the form of anion-selective pores or channels [3,9], similar in at least some respects to anion-selective channels in other cell types [10,11].

The malaria-induced permeation pathways have been suggested to serve a number of roles in the parasitized cell. The normal erythrocyte membrane has limited transport capacity for many of the metabolic and biosynthetic substrates used by the intracellular parasite, and it has been postulated that the new pathways ensure that growth and maturation of the parasite is not rate-limited by the supply of nutrients from the surrounding medium [9,12–14]. The pathways might also be important in mediating the rapid efflux from the infected cell of potentially hazardous products of parasite metabolism. It has been estimated that the rate of production of lactic acid exceeds the lactate transport capacity of the normal erythrocyte [15,16] and that if the parasite is to avoid a build-up of acid it must provide an additional route for the efflux of lactate from the host cell. Similarly, the digestion of haemoglobin by the parasite generates large quantities of free amino acids which, if allowed to accumulate in the host cell, would cause osmotic swelling, leading eventually to haemolysis [1,17]. The induced pathways, by providing a route for the efflux of amino acids, might therefore play a role in host cell volume homeostasis.

If the induced-transport pathways in malaria-infected erythrocytes do play a role in the growth and development of the intracellular parasite, they may be a viable chemotherapeutic target. There is some evidence in support of this view. A number of structurally unrelated reagents that block malaria-induced transport have been shown to inhibit the growth of *P. falciparum* *in vitro*. These include a number of anion transport inhibitors [18], phloridzin and a series of phloridzin derivatives [19,20], several cinnamic acid derivatives [21], the sulphonyl urea, glibenclamide, and the related compound meglitinide [10]. These data are consistent with an important role for induced solute transport in the parasitized cell. However, the anti-malarial activity of these compounds cannot be attributed unequivocally to their effects on induced transport and, for some at least, there is evidence that in addition to blocking induced solute transport they interact with intracellular targets [20]. In any case, irrespective of their mode of action, the concentrations of these compounds required to inhibit induced transport and to exert anti-malarial activity exceed those at which they block important channels and transporters in host tissues, thus precluding their use as anti-malarials *in vivo*.

The most potent inhibitor of malaria-induced transport reported to date is 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) [11]. This compound blocks anion-selective channels as well as other anion transport mechanisms in a variety of animal cells and is one of a large series of arylaminobenzoates that were tested originally for their effects on chloride channels in rabbit kidney [22,23]. In the present study we have investigated the effects of a number of other members of this series on the transport into malaria-infected cells of three structurally unrelated solutes: choline (a univalent cation), thymidine (an

Abbreviations used: DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid; MENB, 2-[2-(4-methoxyphenyl)ethylamino]-5-nitrobenzoic acid; NBMPR, nitrobenzylthioinosine; NDPB, 5-nitro-2-(3,3-diphenylpropylamino)benzoic acid; NPBB, 5-nitro-2-(4-phenylbutylamino)benzoic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; PCMBs, *p*-chloromercuribenzenesulphonic acid.

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electroneutral pyrimidine nucleoside) and lactate (a univalent anion). The most potent inhibitors of induced transport were tested for their effects on parasite growth *in vitro*, and the selectivity of these reagents was investigated by comparing their effects on malaria-induced transport with their effects on a number of important anion transport mechanisms in normal mammalian cells. The data are consistent with the hypothesis that much of the induced transport in infected cells occurs via pathways of a single type and support the view that these pathways might be a viable pharmacological target.

MATERIALS AND METHODS

Materials

[¹⁴C]Choline chloride, [³H]thymidine, sodium L-[¹⁴C]lactate, [¹⁴C]inulin and [³H]hypoxanthine were from Amersham International. Nitrobenzylthioinosine (NBMPR) was a gift from Dr. J. D. Young (Department of Physiology, University of Alberta, Edmonton, Alberta, Canada). 4,4'-Di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS), *p*-chloromercuribenzenesulphonic acid (PCMBS), furosemide and BSA (fraction V; essentially fatty-acid-free) were from Sigma Chemical Co. The 16 arylaminobenzoic acid derivatives used in this study were generously provided by Professor R. Greger (Physiologisches Institut der Albert-Ludwigs-Universität, Freiburg, Germany). The chemical structures of these compounds are shown in Table 1. The numbering system is the same as that used by Wangemann *et al.* [22].

Protein concentrations were measured using the Coomassie Blue-based Bio-Rad Protein Assay with BSA standards.

Cell culture

Human erythrocytes (type O) infected with the ITO4 line of *P. falciparum* [24] were cultured under 1% O₂, 3% CO₂ and 96% N₂ in RPMI 1640 culture medium (Gibco, Glasgow, U.K.) supplemented with Hepes (40 mM), glucose (10 mM), glutamine (2 mM), gentamicin sulphate (25 mg/l) and serum [8.5% (v/v), pooled from different blood donors; Blood Transfusion Service, Bristol, U.K.]. All experiments were carried out using synchronized suspensions of trophozoite-infected cells (approx. 35 h post-invasion; 20–95% parasitaemia), prepared using a combination of sorbitol haemolysis [25] and gelatin flotation [26] as described elsewhere [27].

In experiments comparing uninfected cells with malaria-infected material, erythrocytes (from the same donor) were incubated in parallel with the infected red cell cultures under identical conditions for at least 24 h prior to the experiment. Cell counts were carried out using either an improved Neubauer counting chamber or a Coulter Multisizer. Parasitaemia was estimated from methanol-fixed Giemsa-stained smears.

Radioisotope flux measurements

The unidirectional influx of choline, thymidine and lactate into infected and uninfected erythrocytes was estimated from the uptake of [¹⁴C]choline, [³H]thymidine and L-[¹⁴C]lactate respectively. Experiments were carried out at room temperature (approx. 22 °C) with cells washed ($\times 4$) and then resuspended in either a Hepes-buffered saline (125 mM NaCl, 5 mM KCl, 25 mM Hepes, 5 mM glucose, pH 7.4) or RPMI 1640 (experiment shown in Figure 7 only). The experiments carried out in saline were done under nominally 'zero-trans' conditions, although the presence of glucose in the medium will have presumably resulted in a significant intracellular lactate concentration.

Influx measurements were made under conditions that minimized transport via the normal host erythrocyte transport systems. In choline influx experiments the use of an extracellular choline concentration of 1 mM ensured that the endogenous erythrocyte choline carrier (which has a K_m of around 10 μ M) was fully saturated and therefore contributed little to choline uptake [11]. Thymidine influx via the saturable erythrocyte nucleoside transporter was minimized by the use of 1 mM thymidine together with NBMPR (at either 1 or 20 μ M), a potent inhibitor of the endogenous system [28]. Lactate influx into malaria-infected cells was measured at an extracellular concentration of 1 mM in cells pretreated with 10–20 μ M DIDS to inhibit transport via the erythrocyte band 3 anion exchanger and 0.1 mM PCMBS to block the normal erythrocyte lactate transporter [16]. PCMBS at 0.1 mM had no effect on malaria-induced choline transport. DIDS and NBMPR both caused some inhibition of induced transport; in the paired choline/thymidine/lactate flux experiments in which both DIDS and NBMPR were present (represented in Figures 2 and 3), these agents were therefore used at the relatively low concentrations of 10 and 1 μ M respectively.

In preliminary experiments, choline influx into malaria-infected erythrocytes was found to be linear with time for ≥ 15 min. Throughout this study unidirectional choline influx rates into both infected and uninfected cells were therefore estimated from the uptake of [¹⁴C]choline over a 10–15 min incubation period. For all choline experiments other than those giving rise to Figures 2 and 3, fluxes commenced with the addition of [¹⁴C]choline, together with unlabelled substrate, to a microcentrifuge tube containing cells (with or without the inhibitors of interest). The final sample volume was 0.5 ml and the final cell concentration was typically 2×10^8 cells/ml. Influx was terminated at the appropriate time by transferring aliquots (0.11 ml) of the suspension to microcentrifuge tubes containing 0.8 ml of an ice-cold stopping solution (Hepes-buffered saline containing 1 mM furosemide [11]) layered over 0.25 ml of dibutylphthalate. The tubes were centrifuged immediately (10000 g, 20 s) to sediment the cells below the oil.

The induced transport of thymidine and lactate into malaria-infected erythrocytes is several orders of magnitude faster than that of choline [11]. Influx rates for these substrates were therefore measured using an alternative approach. In time-course experiments (as represented in Figure 1) a series of 6–10 microcentrifuge tubes was prepared, each tube containing 0.15 ml of saline (with radiolabelled substrate, unlabelled substrate and, where appropriate, transport inhibitors) layered over 0.2 ml of dibutylphthalate. The tubes were arranged in a microcentrifuge then, at predetermined intervals, an aliquot of cell suspension was dispensed into each tube in turn, giving a final cell concentration of approx. 2×10^8 cells/ml. Immediately following the addition of cells to the final tube in the series the samples were centrifuged (10000 g, 20 s) to terminate the flux. The time taken between starting the centrifuge and termination of the flux was estimated as 2 s (by extrapolation of data from short time-course experiments).

In later experiments, rates of influx of thymidine and lactate into infected cells were estimated from the amount of [³H]thymidine or L-[¹⁴C]lactate taken up within a fixed 5 s incubation period, using the same technique as in the time-course experiments.

It was found in preliminary experiments that exposure of aqueous solutions of the arylaminobenzoates to a layer of dibutylphthalate led to a time-dependent loss of inhibitory activity from the solution, presumably as a result of these hydrophobic compounds being adsorbed into the oil phase. In all

experiments in which fluxes were carried out using cell suspensions layered over oil, the arylaminobenzoates of interest were therefore added, where required, immediately (5–10 s) prior to beginning the flux by the addition of an aliquot of cells.

In one series of experiments (giving rise to Figures 2 and 3) a quantitative comparison was made of the effects of inhibitors on the transport into infected cells of choline, thymidine and lactate. In view of the effects of the dibutylphthalate layer (used by necessity in the fast thymidine and lactate influx experiments) on the efficacy of the inhibitors, it was necessary to devise a choline influx protocol in which the cell suspension (in the presence or absence of inhibitors) was exposed to a layer of dibutylphthalate for a similar length of time as in the thymidine and lactate experiments. In these experiments choline fluxes were initiated by the addition of an aliquot of cells (0.15 ml) to a saline solution (0.15 ml) containing [^{14}C]choline, unlabelled choline and, where appropriate, arylaminobenzoates (added 5–10 s before the cells), layered over 0.2 ml of dibutylphthalate. After 5 s, 0.2 ml of the suspension was transferred to a second microcentrifuge tube (not containing dibutylphthalate) in which influx was allowed to continue for a further 10–15 min. The flux was terminated by the transfer of 0.15 ml of the suspension to a third tube containing 0.2 ml of dibutylphthalate, followed by centrifugation to separate the cells from the extracellular solution.

In all experiments, following sedimentation of the cells below the oil, the aqueous supernatant solution was removed by aspiration and the radioactivity remaining on the walls of the tube was removed by rinsing the tubes four times with water. The dibutylphthalate was aspirated and then the cell pellet was lysed with 0.1 % (v/v) Triton X-100 (0.5 ml) and deproteinized by the addition of 5 % (w/v) trichloroacetic acid (0.5 ml), followed by centrifugation (10000 *g*, 10 min). Radioactivity was measured using a β -scintillation counter.

In the rapid (thymidine and lactate) influx experiments the amount of radiolabel trapped in the extracellular space within the cell pellets was estimated either using [^{14}C]inulin as an extracellular space marker or from the amount of [^{14}C]thymidine or [^{14}C]lactate in pellets sampled (within < 2 s of combining the cells and the radiolabelled substrate) from suspensions containing 0.1 mM furosemide to block influx [11]. Similar results were obtained using the two different methods. In the slower choline influx experiments, in which choline uptake was monitored over a 10–15 min period, the extracellular space in the cell pellets was estimated from [^{14}C]choline in pellets derived from aliquots of the flux suspension sampled (into microcentrifuge tubes containing 0.8 ml of ice-cold stopping solution layered over 0.25 ml of dibutylphthalate) and centrifuged within a few seconds of adding the [^{14}C]choline to the cells.

In experiments to test the effects of inhibitors on the endogenous erythrocyte lactate transporter, [^{14}C]lactate influx was measured in normal uninfected erythrocytes (pretreated with 20 μM DIDS to inhibit transport via band 3) using procedures similar to those outlined above, with a fixed 10 s incubation period. The effects of inhibitors on the band 3 anion exchanger of uninfected human erythrocytes were tested using $^{35}\text{SO}_4^{2-}$ efflux as a convenient measure of band 3 activity. The $^{35}\text{SO}_4^{2-}$ efflux protocol was modified from that described by Goldstein and Brill [29].

In vitro parasite growth assays

The anti-malarial activity of a number of the arylaminobenzoates was tested using [^3H]hypoxanthine incorporation as a marker for parasite growth [30]. Serial dilutions of the inhibitors in DMSO (0.4 μl) were dispensed into 96-well culture plates to which were

then added tightly synchronized cultures of ring-stage infected erythrocytes (0.2 ml at 2 % haematocrit and 1–3 % parasitaemia in growth medium). [^3H]Hypoxanthine was added at an activity of 2.5 $\mu\text{Ci/ml}$ (specific radioactivity 5 mCi/mmol). The plates were maintained in a gas-tight box under 1 % O_2 , 3 % CO_2 and 96 % N_2 at 37 °C for 25 h so that labelling occurred within a single cycle of maturation, then harvested using an LKB harvester. A flat-bed Betaplate scintillation system was used to measure incorporated radioactivity.

RESULTS

Effects of arylaminobenzoates on induced solute transport

In this study, the rate of choline transport into normal uninfected human erythrocytes presented with an extracellular choline concentration of 1 mM at approx. 22 °C was $1.8 \pm 0.2 \mu\text{mol/h}$ per 10^{12} erythrocytes ($n = 18$; $\pm$ S.E.M.). In erythrocyte suspensions infected with late-stage trophozoites, at parasitaemias ranging from 23 to 78 %, the rate of choline influx ranged from 51 to 168 $\mu\text{mol/h}$ per 10^{12} erythrocytes ($n = 18$). The average rate of choline influx into trophozoite-infected cells, corrected to 100 % parasitaemia, was $88 \pm 7 \mu\text{mol/h}$ per 10^{12} erythrocytes ($n = 18$), almost 50 times the rate in uninfected cells.

Sixteen different arylaminobenzoates were tested for their effects on the influx of choline into malaria-infected erythrocytes. All were found to inhibit the induced transport, with IC_{50} values given in Table 1.

The four most potent inhibitors of malaria-induced choline transport, i.e. NPPB, 5-nitro-2-(4-phenylbutylamino)benzoic acid (NPBB), 2-[2-(4-methoxyphenyl)ethylamino]-5-nitrobenzoic acid (MENB) and 5-nitro-2-(3,3-diphenylpropylamino)benzoic acid (NDPB), were tested for their effects on the malaria-induced transport of two structurally unrelated solutes: thymidine, an electroneutral and non-metabolized pyrimidine nucleoside, and lactate, a univalent anion. Figure 1(a) shows time courses for the influx of thymidine into infected and uninfected cells. The cells were pretreated with NBMPR (20 μM) to inhibit the endogenous erythrocyte nucleoside transporter [28]. Under the conditions of the experiment, thymidine influx into malaria-infected erythrocytes (corrected to 100 % parasitaemia) was $13.5 \pm 2.0 \text{ mmol/h}$ per 10^{12} erythrocytes ($n = 9$), compared with a value of $0.32 \pm 0.05 \text{ mmol/h}$ per 10^{12} erythrocytes ($n = 9$) in uninfected erythrocytes. The four inhibitors (at 1 μM) substantially reduced the initial influx rate.

Corresponding lactate influx time courses are shown in Figure 1(b). In samples pretreated with DIDS (10 μM) and PCMBs (0.1 mM) to inhibit the erythrocyte anion exchanger and the endogenous lactate transporter respectively [16], the rate of lactate influx into uninfected cells was $0.087 \pm 0.009 \text{ mmol/h}$ per 10^{12} erythrocytes ($n = 9$). By contrast, lactate transport into malaria-infected erythrocytes was extremely rapid; the average initial influx rate (corrected to 100 % parasitaemia) was $15.4 \pm 1.0 \text{ mmol/h}$ per 10^{12} erythrocytes ($n = 9$). As in the thymidine experiments, lactate influx into infected cells was slowed by all four of the arylaminobenzoates tested.

Figure 2 shows dose–response curves for the effects of NPPB, NPBB, MENB and NDPB on the transport of choline, thymidine and lactate into *P. falciparum*-infected erythrocytes. For each inhibitor the dose–response curves for the three different substrates were very similar. The IC_{50} values for the curves shown in Figure 2 are a little higher than those reported in Table 1. This is a result of the methodological differences outlined in the Materials and methods section. In the paired experiments giving rise to Figure 2, but not in the experiments from which the data of Table 1 were derived, the cell suspensions to which the

Table 1 IC_{50} values for the inhibition of malaria-induced choline transport by 16 different arylaminobenzoates

The induced choline transport component was calculated by subtracting the flux measured in uninfected cells (cultured in parallel with infected cells) from that measured in infected cells from the same donor in the presence of 0, 0.1, 1, 10, 100 and in some cases 0.01 and/or 500 μ M inhibitor. The IC_{50} values (i.e. the concentrations of inhibitor required to reduce the induced-transport component to 50% of its value in the absence of inhibitor) were calculated from a simple quadratic expression fitted by least-squares regression to the upwardly concave portion of the dose-response curves. The values shown are means $\pm$ S.E.M. from 2–3 experiments, each on cells from a different donor.

Inhibitor	Structure	IC_{50} (μ M)	<i>n</i>
140		2.0 ± 0.1	2
141		2.0 ± 0.3	2
142		0.5 ± 0.3	2
143		0.4 ± 0.1	2
144 (NPPB)		0.14 ± 0.03	3
145 (NPBB)		0.10 ± 0.04	3
146		0.6 ± 0.2	2
148 (MENB)		0.26 ± 0.10	3
149		0.40 ± 0.11	2
150		2.9 ± 1.1	3
151		19 ± 4	2
152		5.1 ± 0.5	2
153		11.1 ± 0.7	2
154		0.6 ± 0.2	2
155 (NDPB)		0.17 ± 0.05	3
156		0.5 ± 0.1	2

Figure 1 Time courses for the influx of (a) the non-metabolized pyrimidine nucleoside thymidine and (b) the monocarboxylate anion lactate into uninfected erythrocytes ($\blacktriangle$) and into malaria-infected erythrocytes in the absence of induced-transport inhibitors ($\bullet$) and in the presence of NPPB ($\triangle$), NPBB ($\square$), MENB ($\circ$) or NDPB (∇)

The inhibitors were each present at a concentration of 1 μ M. The data are averaged from three experiments, each on cells from a different donor, and are corrected to 100% parasitaemia. Error bars denoting $\pm$ S.E.M. are, for clarity, shown only for the time courses measured in the absence of the arylaminobenzoates.

inhibitor had been added were exposed to dibutylphthalate both before and during the fluxes. This exposure caused a slight decrease in the inhibitory potency of the arylaminobenzoates.

The data in Figure 2 are averaged from duplicate experiments on cells from a single donor. Figure 3 shows data averaged from experiments on cells from four different donors, comparing the effects of a 1 μ M concentration of the four inhibitors on the induced transport of choline, thymidine and lactate. For each of the four inhibitors there was no significant difference between their effects on the induced transport of the three different substrates (one-way ANOVA; $P \geq 0.4$).

Comparison of effects of arylaminobenzoates on malaria-induced transport with those on normal mammalian anion transport pathways

The arylaminobenzoates tested here inhibit chloride channels in mammalian cells with varying degrees of potency [22,23]. Figure 4 compares the effects of these compounds on malaria-induced transport with those on chloride channels in rabbit kidney; the IC_{50} values for their effects on malaria-induced choline influx (from Table 1) are plotted against those for inhibition of anion conductance in the basolateral membrane of the thick ascending limb of the loop of Henle (measured by Wangemann et al. [22]). NPPB was a potent inhibitor of both systems. NPBB, MENB

Figure 2 Dose-response curves for the effects of NPPB, NPBB, MENB and NDPB on the malaria-induced transport of choline (○), thymidine (□) and lactate (△) in cells from a single donor

All samples contained 1 μ M NBMPR, 10 μ M DIDS and 0.1 mM PCMBs, together with 1 mM choline, 1 mM thymidine and 1 mM lactate. The malaria-induced transport component for each substrate was calculated by subtracting the flux measured in uninfected cells from that measured in infected cells from the same donor, and is expressed as a percentage of the flux measured in the absence of arylaminobenzoates. Each set of data is averaged from duplicate experiments. The lines were drawn using an equation of the form: Induced flux (% of control) = $(A - D) / (1 + ([inhibitor]/C)^B) + D$, fitted to the data by non-linear least-squares regression.

Figure 3 Effects of NPPB, NPBB, MENB and NDPB on the malaria-induced transport of choline (closed bars), thymidine (open bars) and lactate (shaded bars)

The inhibitors were each present at a concentration of 1 μ M. All samples contained 1 μ M NBMPR, 10 μ M DIDS and 0.1 mM PCMBs, together with 1 mM choline, 1 mM thymidine and 1 mM lactate. The malaria-induced transport component for each substrate was calculated by subtracting the flux measured in uninfected cells from the flux measured in infected cells from the same donor, and is expressed as a percentage of that measured in the absence of arylaminobenzoates. Each set of data is averaged from duplicate experiments on cells from four different donors. For each of the four inhibitors there was no significant difference between the effects on the induced transport of the three different substrates (one-way ANOVA; $P \geq 0.4$).

and NDPB were substantially better inhibitors of the malaria-induced pathway than of anion channels in the mammalian kidney. In particular, MENB inhibited the malaria-induced

Figure 4 Comparison of the effects of arylaminobenzoates on malaria-induced choline transport with their effects on anion channels in the mammalian (rabbit) kidney

The IC_{50} values for the inhibition of anion conductance in the basolateral membrane of the thick ascending limb of the loop of Henle (from [22]) are plotted against those for the inhibition of malaria-induced choline transport. The enantiomeric pair compounds 150 and 151 are shown as open and filled squares respectively, and the enantiomeric pair compounds 152 and 153 as open and filled diamonds respectively.

Figure 5 Comparison of the effects of arylaminobenzoates on malaria-induced choline transport (closed bars) with their effects on band 3-mediated SO_4^{2-} transport (open bars) and on lactate transport (shaded bars) in uninfected erythrocytes

The inhibitors were each present at a concentration of $10 \mu\text{M}$. The choline transport data are averaged from 3–7 experiments, the SO_4^{2-} transport data from 3–4 experiments and the lactate transport data from 3 experiments, each on cells from a different donor.

Figure 6 Dose–response curves for the effects of NPPB (Δ), NPBB ($\square$), MENB ($\circ$) and NDPB (∇) on incorporation of $[^3\text{H}]$ hypoxanthine by malaria parasites over a 25 h period

The $[^3\text{H}]$ hypoxanthine was added to sorbitol-synchronized ring-stage cultures. The growth medium contained 2% (v/v) serum. The data are from a single representative experiment; each point represents the average $[^3\text{H}]$ hypoxanthine incorporation measured in 3–6 replicate wells of a 96-well plate. Error bars ($\pm$ S.D.) fall within the symbols. The lines were drawn using an equation of the form: $[^3\text{H}]$ Hypoxanthine incorporation (% of control) = $(A - D)/(1 + ([\text{inhibitor}]/D)^B) + D$, fitted to the data by non-linear least-squares regression.

pathway with an IC_{50} ($0.26 \mu\text{M}$) more than 350-fold lower than that for its inhibition of the mammalian epithelial anion channels.

In addition to inhibiting anion channels in mammalian cells, NPPB is an effective inhibitor of anion exchangers (homologues of the erythrocyte band 3 protein) in a number of tissues [23] and of the lactate transporter of normal erythrocytes [31]. Figure 5 compares the effects of a number of the arylaminobenzoates on the malaria-induced pathway with their effects on both the band 3 anion exchanger and the lactate transporter of normal human erythrocytes. At a concentration of $10 \mu\text{M}$, NPPB, NPBB and MENB blocked both the malaria-induced pathway and band 3-mediated SO_4^{2-} efflux. NPPB and MENB ($10 \mu\text{M}$) both caused slight inhibition of the lactate transporter, whereas NPBB did not. In the case of the diphenyl derivative, NDPB, a $10 \mu\text{M}$ concentration blocked the malaria-induced pathway while having no significant effect on either band 3-mediated anion exchange or the lactate transporter.

Figure 7 Effects of serum (8.5%, v/v) and of BSA (5 mg/ml) on the inhibition of malaria-induced choline influx by NPPB, NPBB, MENB and NDPB

The inhibitors were each present at a concentration of $10 \mu\text{M}$. Induced choline influx is expressed as a percentage of that measured in the absence of inhibitor in RPMI, RPMI supplemented with 8.5% (v/v) serum or RPMI supplemented with 5 mg/ml albumin. The control and serum data are averaged from 4 experiments and the albumin data from 2 experiments, each on cells from a different donor.

As well as testing the four most potent inhibitors of malaria-induced transport on SO_4^{2-} efflux from normal (uninfected) erythrocytes, we tested the effects of the enantiomeric pair, compounds 150 and 151. Their relative effects on band 3-mediated SO_4^{2-} efflux from normal cells were found to be the reverse of those on the malaria-induced pathway. Compound 151, the *S*-enantiomer, was a more potent inhibitor of SO_4^{2-} efflux than compound 150, the *R*-enantiomer, whereas the converse was true for their relative effects on malaria-induced choline transport (Figure 5).

Effects of arylaminobenzoates on parasite growth

The four most potent inhibitors of induced transport in malaria-infected cells were tested for their effects on parasite growth using a $[^3\text{H}]$ hypoxanthine incorporation assay [30]. In preliminary experiments in which parasites were cultured in medium containing 8.5% (v/v) serum, the inhibitors were found to have little effect at concentrations up to $50 \mu\text{M}$. However, when the serum concentration was reduced to 2% (v/v), $[^3\text{H}]$ hypoxanthine incorporation was inhibited by all four inhibitors (Figure 6). The IC_{50} values for the inhibition of $[^3\text{H}]$ hypoxanthine incorporation were approx. $30 \mu\text{M}$ for NPPB and MENB, $25 \mu\text{M}$ for NPBB and $17 \mu\text{M}$ for NDPB.

The reduced ability of these anion channel blockers to inhibit parasite growth in the presence of 8.5% (v/v) serum [in comparison with their inhibitory effect in the presence of 2% (v/v) serum] suggests that serum components interfered with the interaction of these compounds with the infected cells. This is borne out by the data in Figure 7 which show that inhibition of malaria-induced choline transport by NPPB, NPBB, MENB and NDPB was decreased markedly by the presence of 8.5% (v/v) serum in the external medium. One serum component that is likely to be involved in the attenuation of inhibition is albumin, a protein known to bind large hydrophobic anions (such as the inhibitors tested here). The 8.5% serum solution was found (using a Bio-Rad protein assay) to have a total protein concentration of 5 mg/ml. The inclusion of an equivalent concentration of BSA (in place of serum) in the extracellular medium caused a marked reduction in the efficacy of the four most potent induced-transport blockers, consistent with albumin being at

least in part responsible for the effect of serum on transport inhibition.

DISCUSSION

In this study, 16 different arylaminobenzoates were tested for their effects on the influx of choline into malaria-infected erythrocytes. All were found to inhibit the malaria-induced choline transport component (Table 1). For each of the four most potent inhibitors identified, i.e. NPPB, NPBB, MENB and NDPB, the dose-response curves for their effects on induced choline transport (in cells from a single donor) were very similar to those for their effects on the induced transport of the pyrimidine nucleoside thymidine and of the univalent carboxylate lactate (Figure 2). Similarly, in experiments on cells from four different donors, a comparison of the effects of a $1\text{ }\mu\text{M}$ concentration of each inhibitor on the induced transport of choline, thymidine and lactate showed that for each inhibitor there was no significant difference between the degree of inhibition seen for the three different substrates (Figure 3). These data support the hypothesis that much of the induced transport of a wide range of cations, anions and non-electrolytes into the parasitized erythrocyte is via broad-specificity (anion-selective) pathways of a single type.

All of the inhibitors tested have an anionic ($-\text{COO}^-$) 'headgroup' with an uncharged 'tail' of varying size and polarity. A comparison of the efficacy of compounds 142–145, and of compounds 154 and 155 (Table 1), indicates that their ability to inhibit malaria-induced transport increases with the length and lipophilicity of the hydrophobic tail. This is consistent with the induced pathway having {like anion channels elsewhere (e.g. [32])} hydrophobic components and with the observation that the pathway shows {again, like channels elsewhere (e.g. [32,33])} a higher permeability to hydrophobic solutes than to hydrophilic solutes of similar size [9,11].

The increased permeability of malaria-infected erythrocytes has been suggested to serve a number of important roles in the growth and development of the intracellular parasite, functioning as a nutrient supply route and/or as a route for waste disposal [9,12]. The inhibitors characterized in the present study are the most potent so far identified of malaria-induced solute transport, and the question therefore arises as to whether compounds of this sort might be of value as anti-malarial agents. For any such compound to be of practical use it should block the malaria-induced pathway while not significantly affecting transport pathways in host tissues. The arylaminobenzoates are known to inhibit a number of different mammalian anion transport pathways with varying degrees of potency [23], and it was therefore of interest to compare their effects on malaria-induced transport with those on three representative mammalian systems: the anion channel(s) responsible for the basolateral Cl^- conductance of the thick ascending limb of the loop of Henle in the mammalian kidney, and the lactate transporter and anion exchanger of normal human erythrocytes (both of which have homologues in a wide variety of other tissues).

From Figures 4 and 5 it is clear that there is considerable overlap in the inhibitor-sensitivity of these different pathways. However, a number of the compounds were potent inhibitors of malaria-induced transport while having a much lesser effect on the three different mammalian transport systems. MENB blocked malaria-induced transport at sub-micromolar levels but had little effect on the mammalian Cl^- channel at concentrations below 0.1 mM [22]. NDPB, another potent inhibitor of malaria-induced transport, was a relatively poor inhibitor of Cl^- conductance in the kidney (Figure 4) and, furthermore, had no effect on band 3

or on the erythrocyte lactate transporter, when present at concentrations sufficient to eliminate most of the induced transport of solutes into infected cells.

Among the 16 inhibitors tested were two pairs of enantiomers (compounds 150 and 151, and compounds 152 and 153). For both pairs the *R*-enantiomer (compounds 150 and 152) was a more potent inhibitor of malaria-induced solute transport than the corresponding *S*-enantiomer (compounds 151 and 153). This apparent stereospecificity in the interaction of these inhibitors with the induced-transport pathway would suggest the involvement of protein components in the pathway. It is similar to (though not as pronounced as) that seen for the anion channel in rabbit kidney which was, for both pairs, blocked much more effectively by the *R*-enantiomer than by the *S*-enantiomer [22]. However, this is opposite to what is seen for band 3, which was inhibited more effectively by the *S*-enantiomer, compound 151, than by the *R*-enantiomer, compound 150 (Figure 5). This argues against the induced transport being mediated by a modified form of the band 3 protein and reinforces the point that it is possible to identify compounds that block induced transport while allowing the operation of mammalian anion exchange mechanisms.

In *in vitro* parasite growth assays carried out in the presence of low concentrations of serum, the four most potent transport blockers inhibited [^3H]hypoxanthine incorporation. When the serum concentration was increased the efficacy of the inhibitors decreased, suggesting that serum factors interfered with the interaction of the transport inhibitors with the infected cells. This was confirmed in transport experiments in which it was found that the inhibition of induced choline transport by $10\text{ }\mu\text{M}$ NPPB, NPBB, MENB and NDPB was attenuated in the presence of serum and of the serum component, albumin. Significantly, the diphenyl derivative, NDPB, while not the most potent transport inhibitor in parasitized cells suspended in saline, was the most potent inhibitor of parasite growth (Figure 6) and the most potent inhibitor of transport in the presence of serum (Figure 7). This, together with the observations that it did not inhibit band 3 or the lactate transporter (Figure 5) and that it is a relatively poor inhibitor of Cl^- channels in the kidney (Figure 4), suggests that NDPB may be a useful lead compound in the search for an induced-transport inhibitor of chemotherapeutic value.

Although the finding that the inhibitors identified in this study impede parasite growth is consistent with the induced-transport pathway playing an important role in the development of the intracellular parasite, the anti-malarial activity of these compounds cannot be attributed unequivocally to their effects on induced transport. We cannot exclude the possibility that they have alternative targets within the infected cell. Furthermore, although our results indicate that it is possible to identify compounds that block the induced pathway while not significantly affecting endogenous host anion transport mechanisms, it must be recognized that at high concentrations some of these compounds interfere with metabolic processes in mammalian cells [23]. One approach that might circumvent problems arising from the intracellular toxicity of these reagents is to conjugate them to a hydrophilic moiety, thus allowing them to exert their transport-inhibitory effects at the infected cell surface while not entering normal host cells. It has been shown previously that impermeant macromolecular conjugates of NPPB and a number of other arylaminobenzoates are effective inhibitors of Cl^- channels in cultured epithelial cells [34] and of the erythrocyte anion exchanger [35]. Whether they block the malaria-induced transport pathway (and thereby inhibit the growth of the intracellular parasite) remains to be tested.

In summary, we have tested a series of arylaminobenzoates for their effects on the induced transport of three chemically unrelated solutes into malaria-infected erythrocytes and have identified, among these, the most potent inhibitors so far described of malaria-induced transport. The data provide further support for the view that much of the induced transport of diverse substrates into *P. falciparum*-infected erythrocytes is via pathways of a single type. A comparison of the effects of these inhibitors on malaria-induced transport with their effects on anion channels in rabbit kidney and on the anion exchanger and lactate transporter of normal human erythrocytes showed that the induced-transport pathway interacts with a wider range of inhibitors than these other anion transport mechanisms, and that it is therefore possible to identify compounds that block the malaria-induced pathway while not affecting at least some important transport mechanisms in normal mammalian tissues. Such compounds do exhibit anti-malarial activity *in vitro*. However, their effects on induced transport and on parasite growth are attenuated by serum, at least partly as a result of their being bound by serum proteins such as albumin. If induced-transport inhibitors of this type are to be of value as anti-malarial agents it will therefore be necessary to dissociate the interaction of the inhibitors with the induced-transport pathway from their binding to serum components.

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