y^+LAT-1 mediates transport of the potent and selective iNOS inhibitor, GW274150, in control J774 macrophages

Anwar R Baydoun[*1], Joan Bertran[‡], Shori Thakur[*], John Dawson[†], Manuel Palacín[‡] and Richard G Knowles[†].

[*]School of Life Sciences, University of Hertfordshire, College Lane, Hatfield AL10 9AB, UK. [†]GlaxoSmithKline Research and Development, Stevenage, Hertfordshire, SG1 2NY, UK. [‡]Department of Biochemistry and Molecular Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona.

[1]Author for correspondence: Professor A.R. Baydoun
University of Hertfordshire
Faculty of Health and Human Sciences
School of Life Sciences
College Lane
Hatfield, Herts, AL10 9AB.

Tel: 01707 285 120
Fax: 01707 285 046
e-mail: a.baydoun@herts.ac.uk

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Summary

This study has characterised the transport mechanism(s) for the novel and selective inhibitor of inducible nitric oxide synthase (iNOS), GW274150, in murine macrophage J774 cells. Transport of GW274150 was saturable (K_m = 0.24±0.01 mM and V_{max} of 8.5±0.12 pmol. μg protein^{-1} min^{-1}), pH-insensitive and largely Na^+-independent. Transport was also susceptible to *trans*-stimulation and was significantly inhibited by a 10-fold excess of L-arginine, L-lysine, L-leucine, L-methionine, L-glutamine and 6-diazo-5-oxo-L-norleucine but not by other amino acids or by N-ethylmaleimide. More importantly, the inhibitions caused by the neutral amino acids were critically dependent on Na^+. These results strongly implicate system y^+L in the transport of GW274150. Northern blot analysis confirmed this by revealing the presence of transcripts for y^+LAT-1 but not y^+LAT-2. Thus, taken together, our data show for the first time that J774 macrophages express y^+LAT-1 transporters and that these carriers mediate transport of GW2741500 at least in these cells.

Introduction

Nitric oxide (NO) has emerged as an important mediator of a wide range of physiological and pathophysiological functions in mammalian systems (Moncada *et al.*, 1991; Gross & Wolin, 1995). The precise nature of the responses produced by this diverse molecule appears to depend on the amounts and source of production. Nitric oxide is synthesised by the enzyme nitric oxide synthase (NOS) of which there are at least three distinct isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Knowles & Moncada, 1994; Alderton *et al.*, 2001). Both nNOS and eNOS are Ca^{2+}-calmodulin-dependent enzymes which are constitutively expressed in a variety of tissues and produce short bursts of pico- to nanomolar concentrations of NO following agonist stimulation. At these relatively low concentrations, NO produced by eNOS may function as a physiological mediator, playing an important role in regulating vascular smooth muscle tone, peripheral resistance and thus blood pressure (Rees *et al.*, 1989; Vallance *et al.*, 1989). Similarly, NO from nNOS may mediate diverse neuronal functions acting, for instance, within the peripheral nervous system as a classical neurotransmitter in regulating gastrointestinal motility and regional blood flow (Sanders & Ward, 1992).

In contrast to its constitutive isoforms, iNOS is a Ca^{2+}-calmodulin-insensitive enzyme which is induced following exposure to several inflammatory stimuli including cytokines and bacterial lipopolysaccharide (LPS). Once induced, iNOS produces sustained nano- to micromolar concentrations of NO which contribute to the pathogenesis of various inflammatory diseases. In this regard, enhanced production of NO by iNOS within the vasculature has been implicated in the pathogenesis of septic shock, mediating vascular hyporeactivity not only in experimental models (Julou-Schaeffer *et al.*, 1990) but also in patients with sepsis (Ochoa *et al.*, 1991; Lorente *et al.*, 1993; Tsuneyoshi *et al.*, 1996). Additionally, iNOS derived NO may also play a role in other inflammatory and autoimmune disorders including rheumatoid arthritis (McCartney-Francis *et al.*, 1993) and inflammatory bowel disease (Yamada *et al.*, 1993).

These pathological actions of NO makes selective targeting of iNOS a key therapeutic strategy for the treatment of diseases associated with the over production of NO. Considerable effort has indeed been devoted to this area of NO research, resulting in the

production of a vast and expanding list of compounds which inhibit NO synthesis (Babu & Griffith, 1998; Hobbs *et al.*, 1999; Alderton *et al.*, 2001). Amongst this growing list is (S)-2-amino-(1-iminoethylamino)-5-thioheptanoic acid (GW274150), a sulphur-substituted acetamidine derivative of L-lysine which shows a high selectivity for iNOS (IC_{50} ~1.4 μM) when compared to either eNOS (IC_{50} ~ 466 μM) or nNOS (IC_{50} ~ 145 μM) (Young *et al.*, 2000). Moreover, GW274150 is effective not only against the isolated enzyme but is also highly potent in intact cell systems suggesting that it can be transported into cells to exert its effects.

At present very little is known about the mechanisms associated with the uptake of this compound into cells. We have therefore carried out experiments examining the entry characteristics of GW274150 in an attempt to identify the carrier(s) associated with its transport. These studies are essential and important in terms of the potential therapeutic utility of GW274150. Moreover, the data obtained has identified a novel entry mechanism for iNOS inhibitors which has previously not been described for this class of compounds.

Materials and Methods

Cell culture

The murine monocyte/macrophage cell line J774 was maintained in continuous culture in T75 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units ml^{-1}), streptomycin (100 mg ml^{-1}) and 10% foetal bovine serum. Cells were harvested by gentle scraping and plated into wells for experimentation or passaged every 4 days by dilution of a suspension of the cells 1: 4 in fresh medium.

Measurement of GW274150 transport

J774 cells were plated at a density of 10^5 cells per well in 96-well microtiter plates and allowed to adhere overnight prior to transport studies which were carried out as described previously (Bogle *et al.*, 1992). Briefly, adherent cells were rinsed twice with a modified Hepes-buffered Krebs solution (composition (mM): NaCl, 131; KCl, 5.5; $MgCl_2$, 1; $CaCl_2$, 2.5; $NaHCO_3$, 25; NaH_2PO_4, 1; D-glucose, 5.5; Hepes, 20; pH 7.4) and uptake was initiated by adding Hepes-buffered Krebs (50 µl per well; 37°C) containing [^{14}C]GW274150 (1 µCi ml^{-1}) to the monolayers. Incubations were terminated by placing the plates on melting ice and rinsing cells three times with 200 µl ice-cold Krebs buffer. 100 µl of the bicinchoninic acid protein reagent (Smith *et al.*, 1985) was then added to wells causing rapid lysis of cells. Absorbance values were determined at 620 nm on a Multiskan II plate reader after 40 min of incubation at room temperature. Values obtained were converted to total protein per well using a protein standard curve generated from bovine serum albumin. The amount of radioactivity (dpm) in cell lysates was determined by liquid scintillation counting. Uptake was then calculated and expressed in units of pmoles µg $protein^{-1}$ min^{-1}.

Time course and kinetics of GW274150 transport

The time course of uptake of GW274150 was monitored by incubating cells with [^{14}C]GW274150 (100 µM; 1 µCi ml^{-1}) in Hepes-buffered Krebs solution (50µl; 37°C) for varying time periods ranging from 30 s to 60 min. Transport was then terminated and total [^{14}C]GW274150 accumulated in cells determined as described above.

In kinetic experiments cells were incubated for 1 min in either normal Hepes-buffered Krebs solution (50µl; 37°C) or Na^+-free Krebs solution (see below) containing increasing concentrations (0.005 - 1 mM) of [^{14}C]GW274150 (1 µCiml^{-1}). The data obtained was fitted using the Michaelis-Menten equation or subjected to Eadie-Hofstee analysis.

Selectivity of GW274150 transport

Inhibition of GW274150 transport by competitor amino acids was investigated by incubating cells with Krebs solution containing 100 µM [^{14}C]GW274150 (1 µCi ml^{-1}) in the absence or presence of a 10-fold excess (1 mM) or increasing concentrations of a given inhibitor substrate. Parallel inhibition studies were carried out by replacing normal Krebs solution with Na^+-free Krebs as described below.

pH and Na^+-dependency of GW274150 transport

The dependency of GW274150 uptake on extracellular pH was monitored in Krebs solution titrated with 0.1N HCl or NaOH to achieve pH values ranging from pH 8 to pH 5.5. In sodium-free experiments, the buffer was modified by replacing NaCl, $NaHCO_3$ and NaH_2PO_4 with choline chloride, choline bicarbonate and KH_2PO_4, respectively. All studies were terminated after a 1 min incubation period.

Effects of NEM on GW274150 transport

Cells were incubated with the sulfhydryl reagent, N-ethylmaleimide (NEM) for 1 min to 30 min at 22°C. The effects of NEM were negated by adding 10 mM of 2-mercaptoethanol to each well and the cells washed before monitoring transport of [^{14}C]GW274150 (100 µM; 1 µCiml^{-1}) over 1 min.

Trans-stimulation of GW274150 efflux from [^{14}C]GW274150 loaded J774 cells

Cells were loaded with [^{14}C]GW274150 (100 µM; 1 µCi ml^{-1}) for 30 min at 37°C in standard Krebs buffer. After this period, cells were washed and incubated in fresh buffer containing 1

mM of either L-arginine, L-leucine, L-methionine or L-glutamine. Radioactivity in the medium and in cell lysates was determined at 5, 10 and 15 min and the efflux values expressed as % of radioactivity released from the cells: radioactivity in medium/(radioactivity in medium + radioactivity in lysates)*100%.

Northern Blot analysis of y^+LAT expression

RNA was prepared from confluent monolayers of J774 cells using RNA STAT-60 (Biogenesis, Poole, UK) according to the manufacturer's protocol. The concentration of RNA in solution was determined spectrophotometrically and the quality verified by visualization of 28S and 18S rRNA bands after electrophoresis through a 1% ethidium bromide stained agarose gel. For Northern blot analysis, 25 µg of RNA was electrophoresed through a formaldehyde-agarose gel, transferred to a nylon membrane (Hybond N+, Amersham) and crosslinked by UV irradiation. Probes for y^+LAT-1, y^+LAT-2 and 18S rRNA control were labelled by random-priming using the redi-prime kit from Amersham following the manufacturer's instructions. The y^+LAT-1 probe consisted of a fragment containing nucleotides 28 to 683 of its published sequence (accession number: AJ012754) and the y^+LAT-2 probe consisted of a fragment from nucleotides 142 to 563 of the sequence in the gene bank (accession number: NM_178798). The 18S ribosomal RNA probe corresponded to a fragment encompassing nucleotides 500 to 672 of the rat published sequence (Torczynski *et al.*, 1983). Total RNA isolated from mouse kidney was used as a positive control for identification of y^+LAT1 expression.

After labelling, non-incorporated nucleotides were removed by centrifugation using sepharose spin columns (MicroSpinPM G-25, Amersham). Hybridization was carried out overnight at 65°C in a hybridisation mix that consisted of 0.25 M Na_2HPO_4/NaH2PO$_4$ buffer (pH 7.2), 1mM EDTA, 20% SDS and the respective denatured probes (20 ng at 109 dpm/µg DNA). After hybridization, blots were washed in 2X standard saline citrate buffer (SSC) for 30 minutes at 65°C followed by a second wash in 0.2X SSC for 30 minutes at 65°C. Blots were subsequently exposed to an X-ray film (HyperfilmTM MP, Amersham Pharmacia Biotech) with an intensifying screen at -80°C. The relative intensities of the bands produced were determined by densitometry.

Materials

All reagents for cell culture except foetal calf serum (Sigma) were from Gibco (Paisley, U.K.). Other chemicals were from Sigma or BDH and of the highest grade obtainable. Unlabelled and radioactive L-[^{14}C]GW274150 (54.3 mCi/mmol) were obtained from GlaxoSmithKline (Stevenage, UK) and the 18S ribosomal RNA probe was kindly provided by Dr A. Felipe (University of Barcelona, Spain).

Statistics

All values are means ± s.e.m. of at least three separate experiments with six replicates per experiment. Statistical analyses were performed by one-way ANOVA followed by the Dunnetts multiple comparison test with the overall confidence levels set at 95%.

Results

Time course and Kinetics of GW274150 transport

Time course experiments revealed that transport of L-[^{14}C]GW271450 (100 μM; 1 μCi ml^{-1}) into cells was rapid and linear for up to 3 min, saturating thereafter and reaching a plateau after 20 min of incubation (Fig. 1). On the basis of these observations, all subsequent experiments were carried out over a 1 min period to ensure that transport was measured at initial rates.

Kinetic analysis revealed that transport of GW274150 occurs via a saturable high affinity carrier with a Michaelis constant (K_m) of 0.24±0.01 mM and a maximal transport rate velocity (V_{max}) of 8.5±0.12 pmol. μg protein^{-1} min^{-1} (Fig. 2). Eadie-Hofstee transformation of the data (inset in Fig. 2) produced a single linear regression line compatible with the operation of a single saturable system or systems with indistinguishable functional characteristics. When repeated in the absence of Na^+, transport of GW274150 was indistinguishable to that observed in normal Krebs buffer in that both the V_{max} (8.2±0.11 pmol. μg protein^{-1} min^{-1}) and K_m (0.27±0.01 mM) remained virtually unaltered.

pH and Na^+-dependency of GW274150 transport

The effects of pH on [^{14}C]GW274150 transport was examined in Krebs buffer with varying pH values ranging from pH 8 to pH 6.0. The results in Table 1 show that uptake was largely pH insensitive.

In addition to its pH insensitivity, transport of GW274150 was also largely Na^+-independent since rates measured in Na^+-free Krebs solution were only marginally lower than those determined in the presence of Na^+ (Table 1).

Selectivity of GW274150 transport

Cross inhibition studies were carried out in the presence and absence of Na^+ to evaluate the substrate specificity of the transporter(s) associated with GW274150 uptake by J774 cells. Under these conditions, transport of [^{14}C]GW274150 was not affected by a 10-fold excess of

either 2-methylaminoisobutyric acid (MeAIB), L-alanine, L-valine or ß-2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) but was inhibited by 64±1 %, 60±2 %, 54±2 %, 50±2 %, 45±2 % and 43±2 in the presence of L-arginine, L-lysine, L-leucine, L-methionine, L-glutamine and 6-diazo-5-oxo-L-norleucine (DON) respectively. These inhibitions, with the exception of that caused by L-arginine and L-lysine, were critically dependent on extracellular Na^+ and completely reversed when extracellular Na^+ was replaced with choline (Fig. 3).

Further analysis using selected amino acids show that transport rates for GW274150 were inhibited in a concentration-dependent manner by L-arginine, L-leucine, L-methionine and L-glutamine with respective k_i values of 0.04 mM, 0.06 mM, 0.07 mM and 0.13 mM.

Effects of NEM on GW274150 transport

The profile of inhibition reported above strongly suggests that transport of GW274150 may occur via a system or systems with characteristics similar to those of system y^+ and/or other cationic amino acid transporters such as y^+L/y^+LATs (see Deves & Boyd, 1998; Palacin *et al.*, 1998 for reviews). To further characterise the transporter(s) associated with uptake of GW274150, additional studies were therefore carried out in cells pre-treated with NEM which apparently discriminates between system y^+ and y^+L by inhibiting the former (Deves *et al.*, 1993). In these studies, NEM (0.2 mM) failed to cause any significant inhibition in transport of GW274150 when exposed to cells for short periods of 1 min - 5 min (data not shown). Longer incubations reduced transport of L-arginine but these effects were accompanied by a marked decrease in cell number and total cell protein (~16 % after 10 min and ~37 % after 30 min pre-incubations) which may be indicative of a non-specific cytotoxic action of NEM.

Trans-stimulation of GW274150 transport

Efflux of radioactivity from J774 cells preloaded with [^{14}C]GW274150 was determined over a time period in the absence (control) and presence of 1 mM of either L-arginine, L-leucine or L-glutamine. As shown in Fig. 4, efflux of [^{14}C]GW274150 from control cells was low and showed no significant change over time. In contrast, the presence of either L-arginine, L-

leucine or L-glutamine in the incubation buffer resulted in a significant and time-dependent release of [^{14}C]GW274150 into the medium. Interestingly, L-glutamine which was relatively weaker in inhibiting influx of GW274150 (k_i = 0.13 mM vs 0.04 mM and 0.06 mM for L-arginine and L-leucine respectively) was also less potent in inducing efflux when compared to either L-leucine or L-arginine, indicating that this amino acid may be a relatively weaker substrate for the GW274150 transporter.

Northern blot analysis of y^+LAT expression in J774 cells

To confirm whether J774 macrophages express known system y^+L associated genes, we probed for the expression of y^+LAT-1 and y^+LAT-2 mRNA by Northern blot analysis using total RNA isolated from these cells. The results obtained revealed the expression of transcripts for y^+LAT-1 (Fig 5) but not for y^+LAT-2 (data not shown) in control non-activated cells. These findings are the first, to our knowledge, to report the expression of y^+LAT-1 in J774 macrophages.

Discussion

The characteristics of the transport of GW274150 by J774 macrophages was studied to gain an insight into the mechanisms associated with the uptake of this potent and highly selective iNOS inhibitor into control non-activated cells. The results obtained show that GW274150 can cross cell membranes in a time-dependent manner and that the uptake process is largely pH and Na^+ insensitive. Kinetic analysis revealed a saturable transport system with a relatively high affinity but low capacity. In addition, the transporter was sensitive to *trans*-stimulation but only marginally altered by NEM which was found to be highly cytotoxic, reducing total cell protein significantly even after short incubations.

To further unravel the nature of the transporter(s) involved with the uptake of GW274150, inhibition analysis was carried out using system specific substrates in cross-inhibition studies. The data from these experiments show that uptake can be modified Na^+-independently by cationic amino acids and Na^+-dependently by select neutral amino acids which were also able to induce [^{14}C]GW274150 efflux from cells preloaded with this compound. These findings therefore indicate that transport of GW274150 occurs via a system or systems capable of interacting with both basic and neutral amino acids and thus eliminate other transporters that show selectivity for either neutral or cationic amino acids alone. One such system is the amino acid transporter system A, which is a Na^+-dependent and pH-sensitive carrier of small neutral amino acids (Christensen, 1990; McGivan & Pastor-Anglada, 1994). Conclusive evidence that supports a lack of involvement of this transporter in the uptake of GW274150 is the observation that MeAIB, a model substrate for system A (Christensen, 1965), failed to modify initial rates of GW274150 transport even when used at a 10-fold excess. Furthermore, system A generally shows weak responses to *trans*-stimulation, a key feature of the transport of GW274150 in J774 macrophages. Additionally, L-valine and L-alanine, known substrates for this carrier and indeed the ASC system (Christensen *et al.*, 1967), were also without effect, confirming that neither system A nor ASC may be involved in the transport of GW274150, at least, in J774 macrophages.

The other system for which there is strong evidence against its involvement in the uptake process of GW274150 is the Na^+-insensitive system L, which shows high specificity for branched-chain and aromatic amino acids (Oxender & Christensen, 1963; Christensen, 1990),

and utilises the non-metabolisable bicyclic BCH analogue as a system specific substrate (Christensen *et al.*, 1969). Thus, since BCH was without effect in our studies, it is reasonable to conclude that this pathway does not contribute to the uptake of GW274150 to any significant extent. Interestingly, other known system L substrates such as L-leucine and L-methionine, caused marked inhibition of GW274150 transport. These effects were however critically dependent on the presence of Na^+ which is uncharacteristic of the L transporter whose interaction with both these amino acids is largely Na^+-independent (Oxender & Christensen, 1963; Christensen, 1990). Thus, despite the marked inhibitions caused by L-leucine and L-methionine, the Na^+-dependency of their actions together with the lack of inhibition by BCH strongly argues against system L being utilised by GW274150 for entry into cells.

Similarly, the inhibitions caused by L-glutamine, a system N substrate (Kilberg *et al.*, 1980), and DON, a model substrate for system N (Goldstein, 1975) do not confirm that this pathway is indeed involved in the uptake of GW274150 into J774 macrophages. In this case the argument against is the fact that system N is Na^+-dependent, pH-sensitive and does not interact with either cationic amino acids or leucine (Kilberg *et al.*, 1980). Transport of GW274150 on the other hand, was largely Na^+- and pH-insensitive and was also significantly blocked by both L-arginine and L-lysine and by L-leucine. It is worth noting that L-glutamine, as with L-leucine and L-methionine, is capable of interacting with other amino acid transporters distinct from its recognised carrier (system N) and do so in the absence or presence of Na^+ as a surrogate cation. This mostly occurs through a family of broad-scope carriers capable of transporting both neutral and basic amino acids with relatively high affinity.

At present there are at least three distinct broad-scope amino acid transporters. These systems, identified as $B^{o,+}$, $b^{o,+}$, and y^+L share some common similarities in their characteristics but can also be discriminated on the basis of their requirement for Na^+ in transporting amino acids (Deves & Boyd, 1998; Palacin *et al.*, 1998). For instance, $B^{o,+}$ is defined by Na^+-dependent transport of both neutral and cationic amino acids and also by its ability to accept BCH and interact with relatively small amino acids such as valine (Van Winkle *et al.*, 1985). In contrast, $b^{o,+}$ is Na^+-independent and capable of transporting both

neutral and basic amino acids with comparatively high affinity in the absence of Na^+ (Van Winkle *et al.*, 1988; Bertran *et al.*, 1992; Wells & Hediger, 1992). In our studies transport of GW274150 was largely Na^+-independent and significantly attenuated by both cationic and neutral amino acids with the inhibition caused by the latter being critically dependent on the presence of Na^+ and that caused by the former persisting even on removal of Na^+ from the assay buffer. Thus, on the basis of these findings, it is highly unlikely that either $B^{o,+}$ or $b^{o,+}$ play any significant role in transporting GW274150 into J774 cells. The system that would carry out this process would be one that relies on Na^+ for transport of neutral but not of basic amino acids. This feature is in fact characteristic of the classical broad-scope y^+L transporter (Deves *et al.*, 1992; Deves *et al.*, 1998) which recognises neutral and basic amino acids with high affinity but shows differential effects towards these substrates by its requirement for Na^+ in interacting with the neutral but not with the cationic amino acids (Deves *et al.*, 1992; Angelo & Deves, 1994; Fei *et al.*, 1995; Deves *et al.*, 1998). Moreover, system y^+L does not recognise BCH (Fei *et al.*, 1995), discriminating it from the L transporter and further strengthening its role in the transport of GW274150, at least in J774 macrophages.

In addition to y^+L it is worth considering whether transport of GW274150 may be facilitated by the classical cationic amino acid transporter y^+ (White, 1985; Deves & Boyd, 1998; Palacin *et al.*, 1998). This transport system is know to be expressed in J774 macrophages (Bogle *et al*., 1992; Baydoun *et al*., 1994) and the marked inhibition by both L-arginine and L-lysine together with the Na^+-independency of this inhibition would strongly implicate this system as a key target for the entry of GW274150 into cells. System y^+ however has only weak affinity for some neutral amino acids such as homoserine (Wang *et al.*, 1991) and does not appear to facilitate uptake of either leucine, glutamine or methionine (Kim *et al.*, 1991) which we have shown to be able to cause marked inhibitions of the transport of GW274150 in our system. Also of significance is the additional finding with DON which does not interact with system y^+ in J774 macrophages (Baydoun *et al.*, 1994) but attenuated GW274150 uptake quite markedly in the same cells. The rational deduction from these data therefore is that system y^+, although potentially expressed, may not be the transporter for GW274150 in control J774 cells. This is interesting because GW274150 is an acetamidine derivative of hetero-substituted lysine (Young *et al.,* 2000) and would be expected to interact with system y^+. The fact that this was not the case raises the question of the relative

expression levels of the system y^+ protein in our cells. We cannot rule out that this transporter, although present, may be expressed in relatively low levels that may not be sufficient to make a major contribution to the total uptake of GW274150. Additional studies looking at the expression levels of CAT-1 expression for instance may help shed more light on this. Such studies are however limited by the lack of readily available selective antibodies for these proteins.

Despite the argument raised above, it is clear that system y^+ may not be involved in the transport of GW274150 under our current experimental conditions. Further evidence in support of this notion was obtained in studies with NEM which apparently discriminates between systems y^+L and y^+ by inhibiting the activity of the latter (Deves *et al.*, 1993). This compound however failed to cause any discernible change in the transport of GW274159 when used under non-cytotoxic conditions (short incubations) at concentrations (0.2 mM) shown to abolish y^+ activity in other systems (Deves *et al.*, 1993; Patel *et al.*, 1996; Peter *et al.*, 1999; Dall_Asta *et al.*, 2000). Although greater inhibitions were observed with prolonged incubations (> 10 min) these effects were characterised by a conspicuous decrease in cell number and total cell protein, indicative of a non-selective cytotoxic action by NEM. These responses should therefore be interpreted with caution and not ascribed to the expected inactivation of the y^+ transporter defined in other systems.

All the data discussed thus far give a strong indication that y^+L rather than systems A, L, N or y^+ may be the relevant transporter for GW274150 in J774 macrophages. We cannot however rule out that a fraction of GW274150 transport may occur through low affinity routes which are yet to be identified. This supposition is based on the fact that none of the inhibitor amino acids used completely suppressed GW274150 transport into cells. In each case there was a small component of entry which was not susceptible to inhibition even in the presence of a large excess of competitor substrate.

Although the indication is that a transporter similar to y^+L may be involved with uptake of GW274150, it is not clear whether this is the classical y^+L carrier identified in erythrocytes (Deves *et al.*, 1992). The affinity of the latter for amino acids seems somewhat higher (K_m ~ 12 μM) than that inferred in this study for the same amino acids from inhibition studies (K_i

40-130 μM). y^+L is however emerging as a family of distinct heterodimeric proteins which, although characterised by their dependency on Na^+ for transport of neutral amino acids and Na^+-independency for interaction with basic amino acids, may be distinguished by their tissue distribution and by their relative affinity for different substrates. At present there are at least two distinct y^+L transporters which consist of the heavy chain 4F2hc glycoprotein and one of two light chains designated as y^+LAT-1 and y^+LAT-2 (Torrents *et al.*, 1998; Pfeiffer *et al.*, 1999; Broer *et al.*, 2000). Whether these novel proteins are associated with the reported y^+L activity in erythrocytes remains to be established. However, both y^+LAT-1 and y^+LAT-2 are expressed in most tissues with levels of the former being most apparent in the intestine, kidney, lung and leucocytes(Torrents *et al.*, 1998; Pfeiffer *et al.*, 1999) whilst y^+LAT-2 appears to be more prominent in the brain, heart, testis, small intestine and parotids (Broer *et al.*, 2000).

At the functional level, y^+LAT-1 shows a much higher affinity for neutral amino acids (K_m ~20-30 μM vs K_m of ~ 340 μM for L-arginine (Pfeiffer *et al.*, 1999)) while y^+LAT-2 shows a marginal preference for cationic substrates (K_m for L-arginine ~120 μM vs 236-295 μM for leucine and glutamine respectively (Broer *et al.*, 2000)). Either of these carriers may be involved with the transport of GW274150 in J774 macrophages. However, although there are reports of system y^+L activity in rabbit (Racké *et al*., 1998) and rat alveolar macrophages (Messeri Dreißig *et al*., 2000) and y^+LAT-1 expression has been shown in human monocytes (Rotoli *et al*., 2004) there is no published literature on the expression or function of these proteins in J774 macrophages. We have therefore extended our studies by examining whether transcripts for y^+LAT-1 and/or y^+LAT-2 are expressed in our cell system by Northern blot analysis of total RNA isolated from control cells. The data obtained revealed basal expression of y^+LAT-1 mRNA but not of y^+LAT-2, suggesting that the former may be the carrier for GW274150 in J774 macrophages.

In summary data has been produced showing that GW274150 is transported by J774 macrophages via a system which is characteristically similar to the classical system y^+L and in particular to the newly identified y^+LAT-1 transporter. This conclusion is based on several observations including the detection of transcripts for y^+LAT-1 in control cells. Moreover, the characteristic Na^+-dependent and Na^+-independent inhibitions caused by neutral and basic

amino acids respectively, and the insensitivity of the system to pH and NEM together with the *trans*-stimulation of GW274150 efflux by both neutral and cationic amino acids are compatible with the involvement of system y^+L in facilitating transport of GW274150, at least in J774 macrophages. To our knowledge, this is also the first report to demonstrate the expression of y^+LAT-1 in J774 macrophages and, in agreement with a recent report by Rotoli *et al.* (2005), suggests a critical role for these carriers in the transport of NOS inhibitors which, previously, may have been ascribed to the CATs (Baydoun and Mann, 1994). Should y^+LAT-1 be sensitive to induction by inflammatory mediators the resulting upregulation in its expression may provide a mechanism for enhancing the delivery of GW274150 into cells expressing iNOS. This has indeed been shown for transport of other NOS inhibitors through system y^+ (Baydoun and Mann, 1994) and could have potential therapeutic benefits in enhancing the potency of GW274150 against iNOS.

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Table 1. Effects of Na^+ and pH on GW274150 transport in J774 macrophages.

Condition	Transport Rate (pmoles μg protein^{-1} min^{-1})
Na^+-dependency	
Control (+Na^+)	2.1 ± 0.13
-Na^+	1.7 ± 0.07
pH-dependency	
pH 8.0	1.92 ± 0.02
pH 7.4 (Control)	2.05 ± 0.09
pH 7.0	1.99 ± 0.04
pH 6.5	1.92 ± 0.05
pH 6.0	1.84 ± 0.01

Transport of 100 μM GW274150 was measured over 1 min in the presence (normal Krebs solution) or absence of Na^+ and at varying pH values ranging from pH 8-pH 6.0. Values are the means ± s.e.m. of three different experiments with six replicates in each.

Figure 1

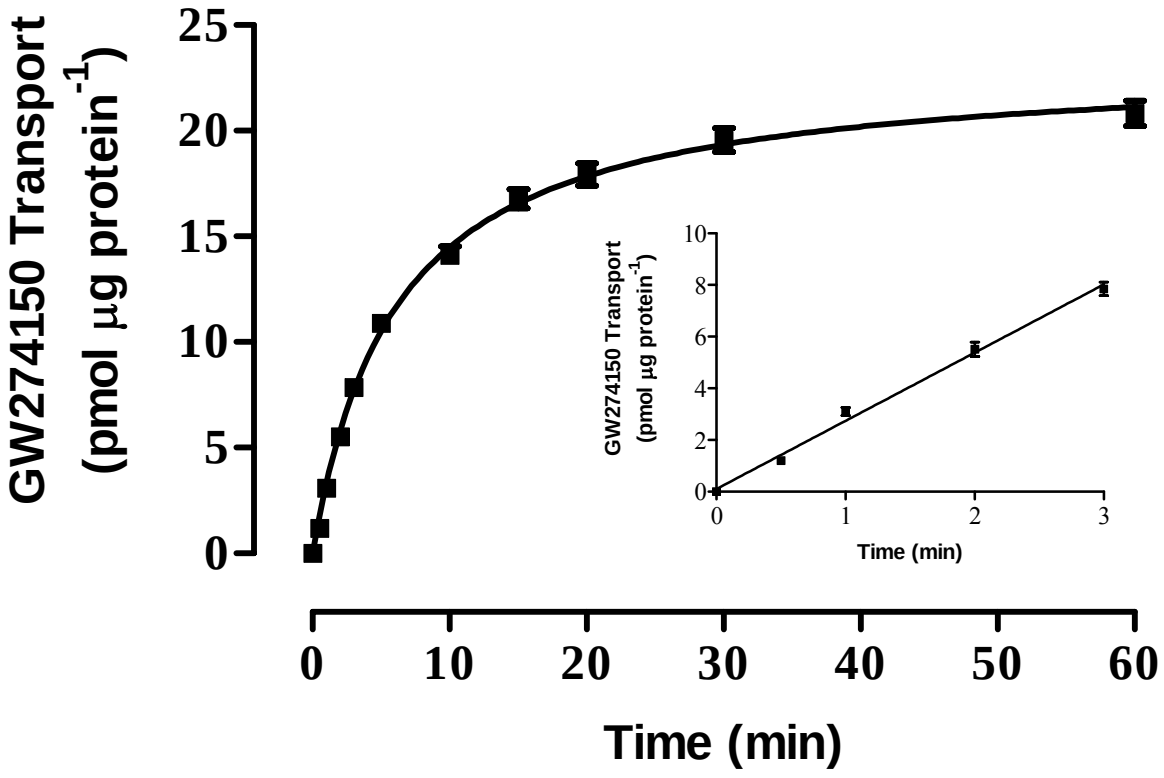
GW274150 Transport
(pmol µg protein-1)
25
20
15
10
5
0
0
10
20
30
40
50
60
Time (min)
GW274150 Transport
(pmol µg protein-1)
10
8
6
4
2
0
0
1
2
3
Time (min)

Figure 2

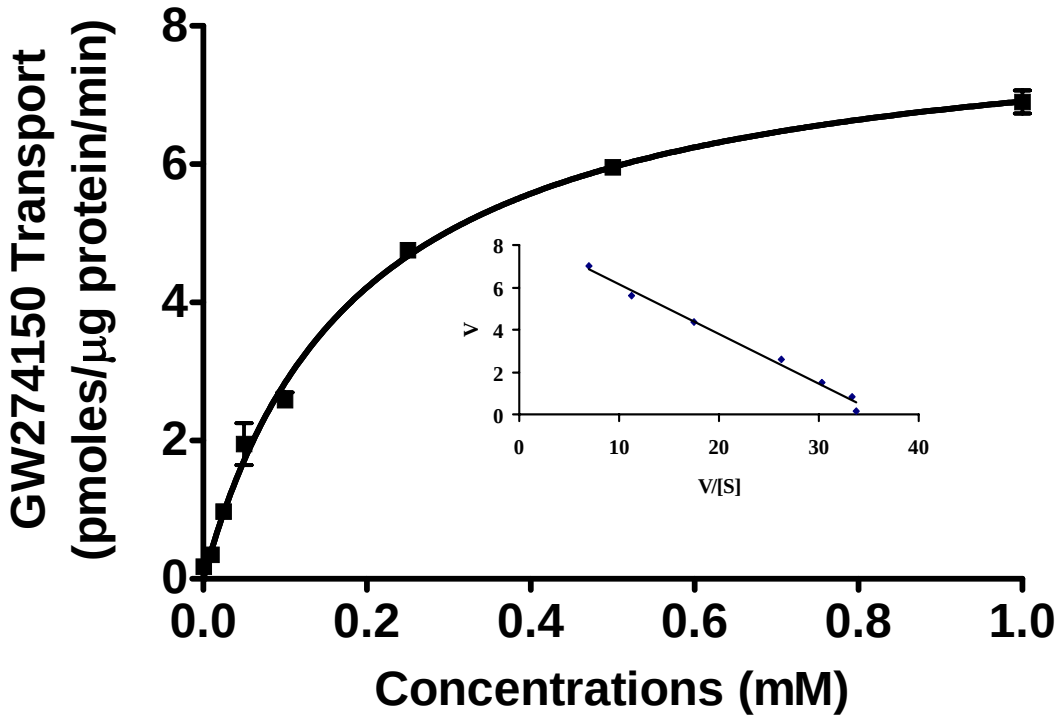
GW274150 Transport
(pmoles/µg protein/min)
8
6
4
2
0
0.0
0.2
0.4
0.6
0.8
1.0
Concentrations (mM)
V
8
6
4
2
0
0
10
20
30
40
V/[S]

Figure 3

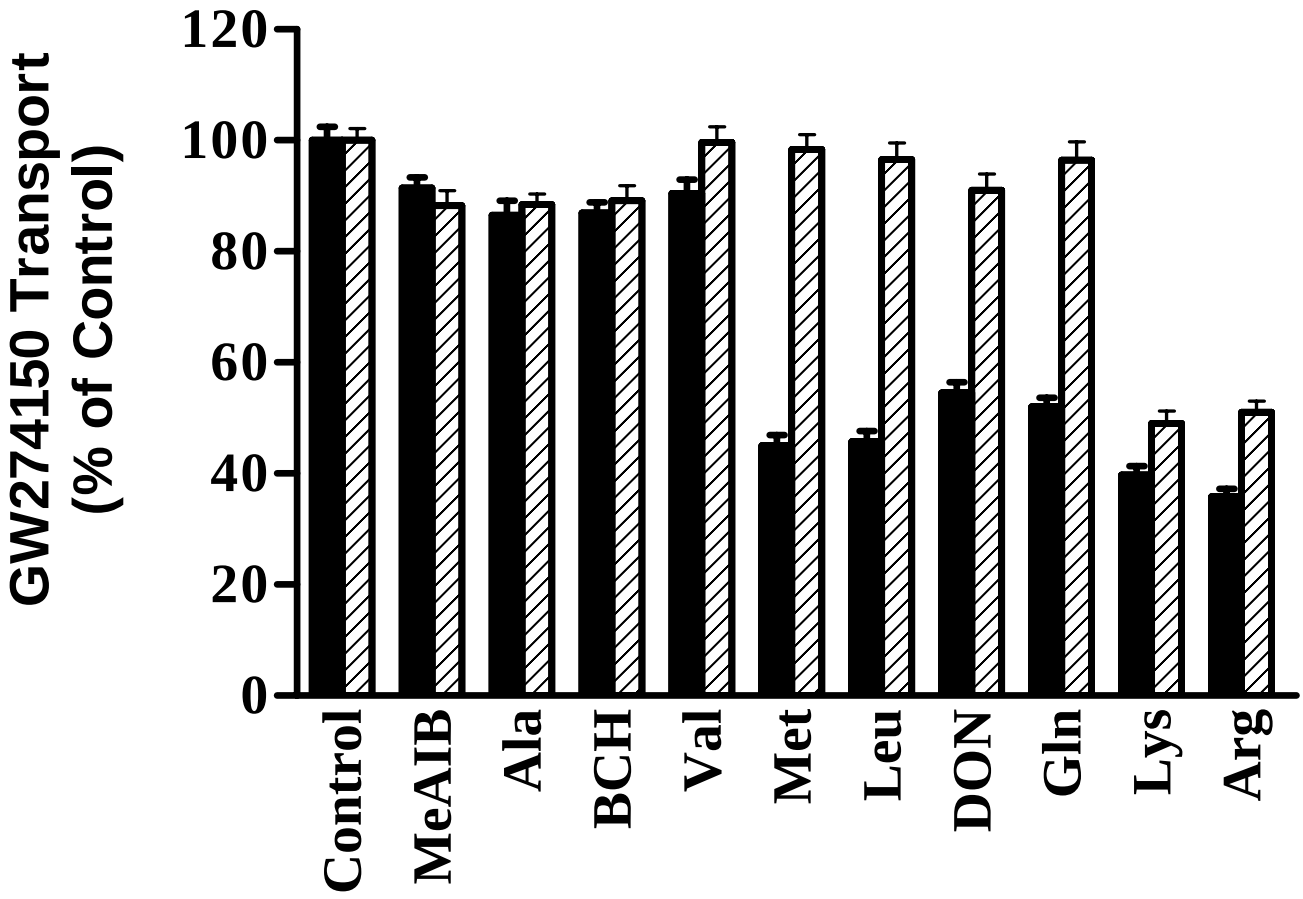
GW274150 Transport
(% of Control)
120
100
80
60
40
20
0
Control
MeAIB
Ala
BCH
Val
Met
Leu
DON
Gln
Lys
Arg

Figure 4

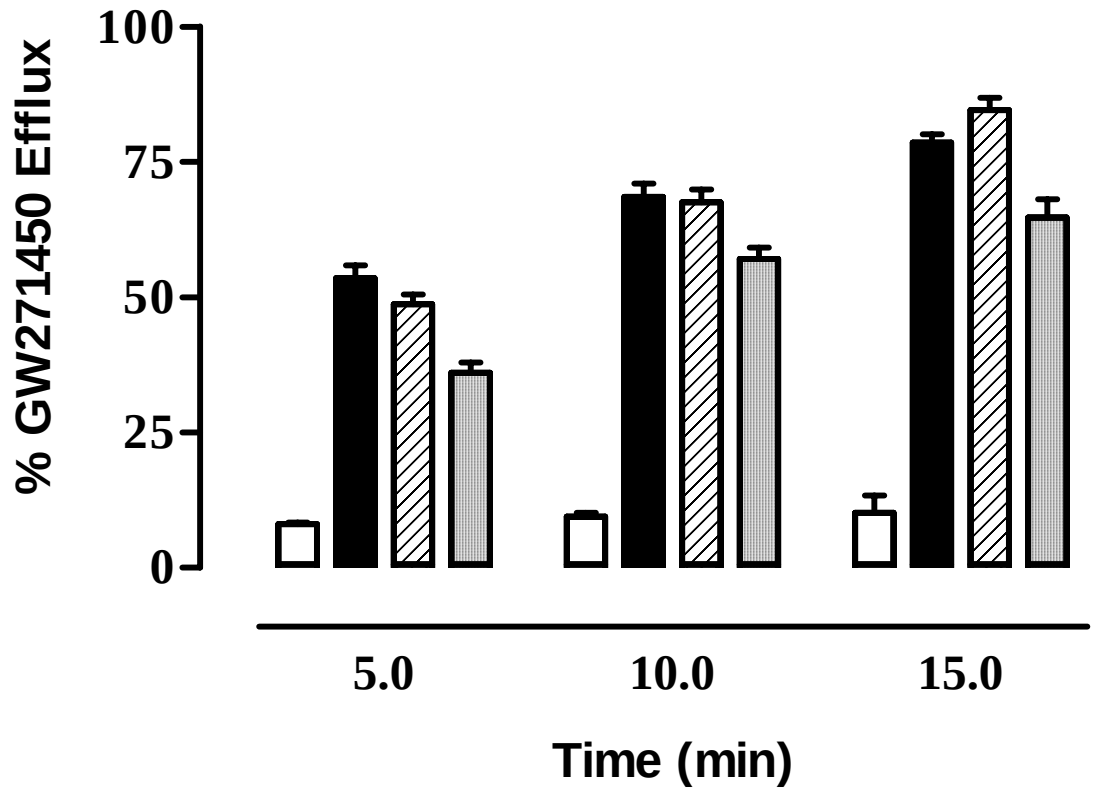

Figure 5

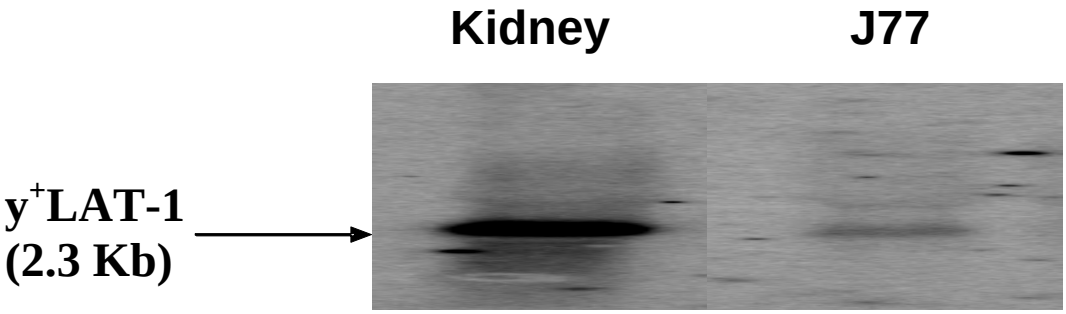

FIGURE LEGENDS

Fig. 1 Time course of GW274150 transport in control J774 macrophages.

Uptake of GW274150 (100 μM, 1 μCi ml^{-1}) was monitored from 30 s to 60 min in normal Krebs buffer as described in methods. The inset shows linear uptake for up to 3 min. Values are the mean ± s.e.m. of 6 replicate measurements in 3 separate experiments.

Fig. 2 Kinetics of GW274150 transport in control J774 macrophages.

Transport of GW274150 (25-1000 μM, 1 μCi ml^{-1}) was monitored over a 1 min period in control cells as described in methods. Values are the mean ± s.e.m. of 6 replicate measurements in 3 separate experiments. The inset shows an Eadie-Hofstee plot of saturable transport.

Fig. 3 Specificity of GW274150 transport in control J774 macrophages.

Inhibition of GW274150 (100 μM, 1 μCi ml^{-1}) transport by a 1 mM excess of competing substrates was measured in the presence (solid bars) and absence (hatched bars) of Na^+ in control non-activated cells. Data are expressed as a percentage of the transport rate measured in the absence of an inhibitor. Abbreviations denote standard amino acid nomenclature. Values denote the mean ± s.e.m. of 6 replicate measurements in 3 experiments.

Fig. 4 Trans-stimulation of GW274150 efflux in control J774 macrophages.

Confluent monolayers of J774 cells were preloaded with GW274150 (100 μM; 1μCiml^{-1}) for 30 min at 37^{o}C in standard Krebs buffer. Efflux of [^{14}C] was determined over a time period in the absence (open bars) and presence of 1 mM of either L-arginine (solid bars), L-leucine (hatched bars) or L-glutamine (spotted bars) as described in methods. Radioactivity in the medium and in cell lysates were determined and the values expressed as % of radioactivity: radioactivity in medium/(radioactivity in medium + radioactivity in lysates)*100%. Data are the mean ± s.e.m. of 6 replicate measurements in 3 experiments.

Fig. 5 Northern blot analysis of y^+LAT-1 mRNA expression in J774 macrophages.

Total RNA from J774 cells or mouse kidney (25 μg) was separated on a 1% agarose gel, blotted to a nylon membrane, hybridized to a ^{32}P labelled probe and developed as described in the methods. The blot shown is representative of 3 independent analyses and 5 independent RNA preparations.