

## **y<sup>+</sup> LAT-1 mediates transport of the potent and selective iNOS inhibitor, GW274150, in control J774 macrophages**

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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 ( $K_m = 0.24 \pm 0.01$  mM and  $V_{max}$  of  $8.5 \pm 0.12$  pmol ·  $\mu$ g protein<sup>-1</sup> min<sup>-1</sup>), pH-insensitive and largely Na<sup>+</sup>-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<sup>+</sup>. These results strongly implicate system y<sup>+</sup>L in the transport of GW274150. Northern blot analysis confirmed this by revealing the presence of transcripts for y<sup>+</sup>LAT-1 but not y<sup>+</sup>LAT-2. Thus, taken together, our data show for the first time that J774 macrophages express y<sup>+</sup>LAT-1 transporters and that these carriers mediate transport of GW274150 at least in these cells.

**Keywords:** GW274150 – J774 macrophages – System y<sup>+</sup> – y<sup>+</sup>LAT-1 – Nitric oxide – Inducible nitric oxide synthase – Amino acid transport

### **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 and 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 and Moncada, 1994; Alderton et al., 2001). Both nNOS and eNOS are Ca<sup>2+</sup>-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 and Ward, 1992).

In contrast to its constitutive isoforms, iNOS is a Ca<sup>2+</sup>-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 and 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 ( $IC_{50} \sim 1.4 \mu M$ ) when compared to either eNOS ( $IC_{50} \sim 466 \mu M$ ) or nNOS ( $IC_{50} \sim 145 \mu M$ ) (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 ( $100 \text{ units ml}^{-1}$ ), streptomycin ( $100 \text{ mg ml}^{-1}$ ) 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 ( $50 \mu l$  per well;  $37^\circ C$ ) containing [ $^{14}C$ ]GW274150 ( $1 \mu Ci \text{ ml}^{-1}$ ) to the monolayers. Incubations were terminated by placing the plates on melting ice and rinsing cells three times with  $200 \mu l$  ice-cold Krebs buffer.  $100 \mu l$  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  $\text{pmoles } \mu g \text{ protein}^{-1} \text{ min}^{-1}$ .

### Time course and kinetics of GW274150 transport

The time course of uptake of GW274150 was monitored by incubating cells with [ $^{14}C$ ]GW274150 ( $100 \mu M$ ;  $1 \mu Ci \text{ ml}^{-1}$ ) in Hepes-buffered Krebs solution ( $50 \mu l$ ;  $37^\circ C$ ) 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 ( $50 \mu l$ ;  $37^\circ C$ ) or  $Na^+$ -free Krebs solution (see below) containing increasing concentrations ( $0.005$ – $1 \text{ mM}$ ) of [ $^{14}C$ ]GW274150 ( $1 \mu Ci \text{ ml}^{-1}$ ). 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  $100 \mu M$  [ $^{14}C$ ]GW274150 ( $1 \mu Ci \text{ ml}^{-1}$ ) in the absence or presence of a 10-fold excess ( $1 \text{ mM}$ ) 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  $0.1 N$  HCl or NaOH to achieve pH values ranging from pH 8 to pH 6. 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  $22^\circ C$ . The effects of NEM were negated by adding  $10 \text{ mM}$  of 2-mercaptoethanol to each well and the cells washed before monitoring transport of [ $^{14}C$ ]GW274150 ( $100 \mu M$ ;  $1 \mu Ci \text{ ml}^{-1}$ ) over 1 min.

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

Cells were loaded with [ $^{14}C$ ]GW274150 ( $100 \mu M$ ;  $1 \mu Ci \text{ ml}^{-1}$ ) for 30 min at  $37^\circ C$  in standard Krebs buffer. After this period, cells were washed and incubated in fresh buffer containing  $1 \text{ mM}$  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:  $\text{radioactivity in medium} / (\text{radioactivity in medium} + \text{radioactivity in lysates}) \times 100\%$ .

### 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,  $25 \mu g$  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  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  buffer (pH 7.2), 1 mM EDTA, 20% SDS and the respective denatured probes (20 ng at 109 dpm/ $\mu\text{g}$  DNA). After hybridization, blots were washed in  $2\times$  standard saline citrate buffer (SSC) for 30 minutes at 65 °C followed by a second wash in  $0.2\times$  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.

### Sources

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-[ $^{14}\text{C}$ ]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).

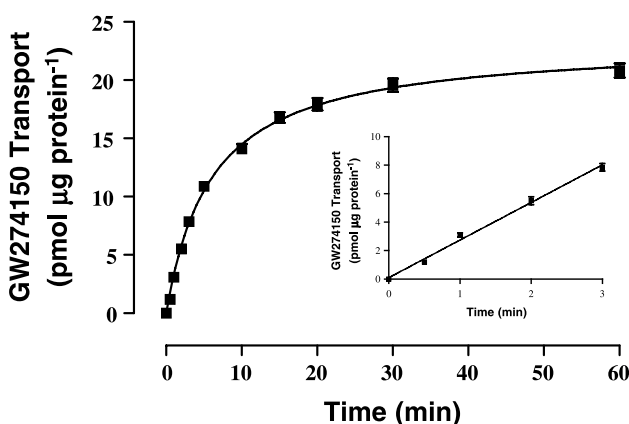
### Statistics

All values are means  $\pm$  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 Dunnett's multiple comparison test with the overall confidence levels set at 95%.

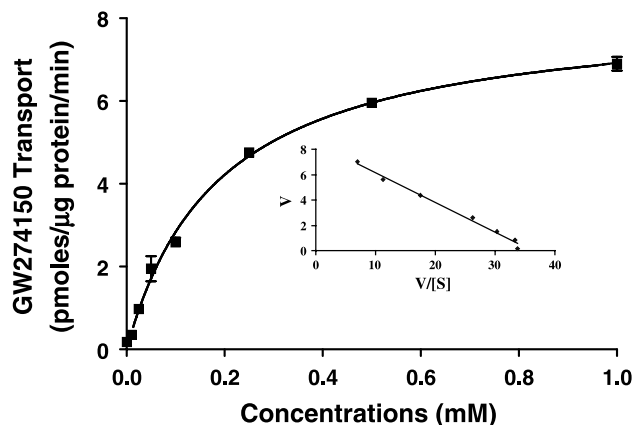
## Results

### Time course and kinetics of GW274150 transport

Time course experiments revealed that transport of L-[ $^{14}\text{C}$ ]GW274150 (100  $\mu\text{M}$ ; 1  $\mu\text{Ci ml}^{-1}$ ) into cells was rapid and linear for up to 3 min, saturating thereafter and reaching a plateau after 20 min of incubation (Fig. 1).



**Fig. 1.** Time course of GW274150 transport in control J774 macrophages. Uptake of GW274150 (100  $\mu\text{M}$ , 1  $\mu\text{Ci ml}^{-1}$ ) was monitored from 30 sec to 60 min in normal Krebs buffer as described in Materials and methods. The inset shows linear uptake for up to 3 min. Values are the mean  $\pm$  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  $\mu\text{M}$ , 1  $\mu\text{Ci ml}^{-1}$ ) was monitored over a 1 min period in control cells as described in Materials and methods. Values are the mean  $\pm$  s.e.m. of 6 replicate measurements in 3 separate experiments. The inset shows an Eadie-Hofstee plot of saturable transport

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  $0.24 \pm 0.01$  mM and a maximal transport rate velocity ( $V_{\max}$ ) of  $8.5 \pm 0.12$  pmol  $\cdot$   $\mu\text{g protein}^{-1} \text{ min}^{-1}$  (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  $\text{Na}^+$ , transport of GW274150 was indistinguishable to that observed in normal Krebs buffer in that both the  $V_{\max}$  ( $8.2 \pm 0.11$  pmol  $\cdot$   $\mu\text{g protein}^{-1} \text{ min}^{-1}$ ) and  $K_m$  ( $0.27 \pm 0.01$  mM) remained virtually unaltered.

### pH and $\text{Na}^+$ -dependency of GW274150 transport

The effects of pH on [ $^{14}\text{C}$ ]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  $\text{Na}^+$ -independent since rates measured in  $\text{Na}^+$ -free Krebs solution were only marginally lower than those determined in the presence of  $\text{Na}^+$  (Table 1).

### Selectivity of GW274150 transport

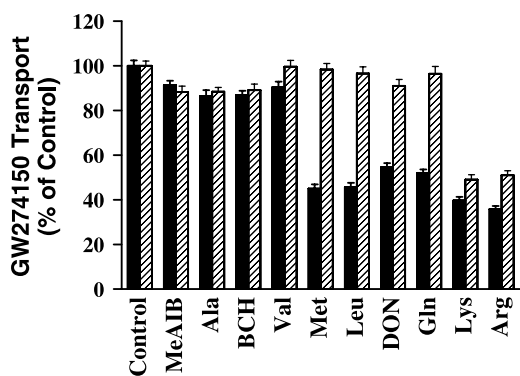
Cross inhibition studies were carried out in the presence and absence of  $\text{Na}^+$  to evaluate the substrate specificity of the

**Table 1.** Effects of  $\text{Na}^+$  and pH on GW274150 transport in J774 macrophages

Condition	Transport rate (pmoles $\mu\text{g}$ protein $^{-1}$ min $^{-1}$ )
<i><math>\text{Na}^+</math>-dependency</i>	
Control (+ $\text{Na}^+$ )	$2.1 \pm 0.13$
– $\text{Na}^+$	$1.7 \pm 0.07$
<i>pH-dependency</i>	
pH 8.0	$1.92 \pm 0.02$
pH 7.4 (control)	$2.05 \pm 0.09$
pH 7.0	$1.99 \pm 0.04$
pH 6.5	$1.92 \pm 0.05$
pH 6.0	$1.84 \pm 0.01$

Transport of 100  $\mu\text{M}$  GW274150 was measured over 1 min in the presence (normal Krebs solution) or absence of  $\text{Na}^+$  and at varying pH values ranging from pH 8–pH 6.0. Values are the means  $\pm$  s.e.m. of three different experiments with six replicates in each

transporter(s) associated with GW274150 uptake by J774 cells. Under these conditions, transport of [ $^{14}\text{C}$ ]GW274150 was not affected by a 10-fold excess of either 2-methyl-aminoisobutyric acid (MeAIB), L-alanine, L-valine or  $\beta$ -2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) but was inhibited by  $64 \pm 1\%$ ,  $60 \pm 2\%$ ,  $54 \pm 2\%$ ,  $50 \pm 2\%$ ,  $45 \pm 2\%$  and  $43 \pm 2\%$  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  $\text{Na}^+$  and completely reversed when extracellular  $\text{Na}^+$  was replaced with choline (Fig. 3).



**Fig. 3.** Specificity of GW274150 transport in control J774 macrophages. Inhibition of GW274150 (100  $\mu\text{M}$ , 1  $\mu\text{Ci ml}^{-1}$ ) transport by a 1 mM excess of competing substrates was measured in the presence (solid bars) and absence (hatched bars) of  $\text{Na}^+$  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  $\pm$  s.e.m. of 6 replicate measurements in 3 experiments

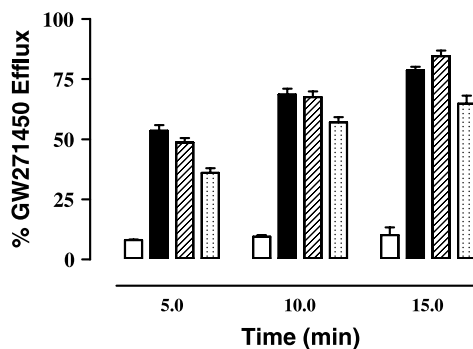
Further analysis using selected amino acids show that transport rates for GW274150 were inhibited in a concentration-dependent manner by L-arginine, L-leucine, L-methionine and L-glutamine with respective  $k_i$  values of 0.04 mM, 0.06 mM, 0.07 mM and 0.13 mM.

#### *Effects of NEM on GW274150 transport*

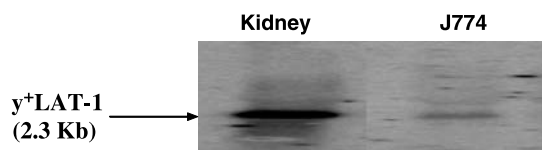
The profile of inhibition reported above strongly suggests that transport of GW274150 may occur via a system or systems with characteristics similar to those of system  $y^+$  and/or other cationic amino acid transporters such as  $y^+\text{L}/y^+\text{LATs}$  (see Deves and Boyd, 1998; Palacin et al., 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  $y^+\text{L}$  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 ( $\sim 16\%$  after 10 min and  $\sim 37\%$  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 [ $^{14}\text{C}$ ]GW274150 was determined over a time period in



**Fig. 4.** Trans-stimulation of GW274150 efflux in control J774 macrophages. Confluent monolayers of J774 cells were preloaded with GW274150 (100  $\mu\text{M}$ ; 1  $\mu\text{Ci ml}^{-1}$ ) for 30 min at  $37^\circ\text{C}$  in standard Krebs buffer. Efflux of [ $^{14}\text{C}$ ] 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 Materials and 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)  $\times 100\%$ . Data are the mean  $\pm$  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  $\mu$ g) was separated on a 1% agarose gel, blotted to a nylon membrane, hybridized to a  $^{32}$ P labelled probe and developed as described in Materials and methods. The blot shown is representative of 3 independent analyses and 5 independent RNA preparations

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  $\text{Na}^+$  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  $\text{Na}^+$ -independently by cationic amino acids and  $\text{Na}^+$ -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  $\text{Na}^+$ -dependent and pH-sensitive carrier of small neutral amino acids (Christensen, 1990; McGivan and 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 et al., 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  $\text{Na}^+$ -insensitive system L, which shows high specificity for branched-chain and aromatic amino acids (Oxender and 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  $\text{Na}^+$  which is uncharacteristic of the L transporter whose interaction with both these amino acids is largely  $\text{Na}^+$ -independent (Oxender and Christensen, 1963; Christensen, 1990). Thus, despite the marked inhibitions

caused by L-leucine and L-methionine, the  $\text{Na}^+$ -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  $\text{Na}^+$ -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  $\text{Na}^+$ - 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 does so in the absence or presence of  $\text{Na}^+$  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  $\text{B}^{\circ,+}$ ,  $\text{b}^{\circ,+}$ , and  $\text{y}^+\text{L}$  share some common similarities in their characteristics but can also be discriminated on the basis of their requirement for  $\text{Na}^+$  in transporting amino acids (Deves and Boyd, 1998; Palacin et al., 1998). For instance,  $\text{B}^{\circ,+}$  is defined by  $\text{Na}^+$ -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,  $\text{b}^{\circ,+}$  is  $\text{Na}^+$ -independent and capable of transporting both neutral and basic amino acids with comparatively high affinity in the absence of  $\text{Na}^+$  (Van Winkle et al., 1988; Bertran et al., 1992; Wells and Hediger, 1992). In our studies transport of GW274150 was largely  $\text{Na}^+$ -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  $\text{Na}^+$  and that caused by the former persisting even on removal of  $\text{Na}^+$  from the assay buffer. Thus, on the basis of these findings, it is highly unlikely that either  $\text{B}^{\circ,+}$  or  $\text{b}^{\circ,+}$  play any significant role in transporting GW274150 into J774 cells. The system that would carry out this process would be one that relies on  $\text{Na}^+$  for transport of neutral but not of basic amino acids. This feature is in fact characteristic of the classical broad-scope  $\text{y}^+\text{L}$  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  $\text{Na}^+$  in interacting with the neutral but not with the cationic amino acids (Deves et al., 1992; Angelo and Deves, 1994; Fei et al., 1995; Deves et al., 1998). Moreover, system  $\text{y}^+\text{L}$  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  $\text{y}^+\text{L}$  it is worth considering whether transport of GW274150 may be facilitated by the classical cationic amino acid transporter  $\text{y}^+$  (White, 1985; Deves and Boyd, 1998; Palacin et al., 1998). This transport system is known 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  $\text{Na}^+$ -independency of this inhibition would strongly implicate this system as a key target for the entry of GW274150 into cells. System  $\text{y}^+$  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  $\text{y}^+$  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  $\text{y}^+$ , 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  $\text{y}^+$ . The fact that this was not the case raises the question of the relative expression levels of the system  $\text{y}^+$  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  $\text{y}^+$  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  $\text{y}^+\text{L}$  and  $\text{y}^+$  by inhibiting the activity of the latter (Deves et al., 1993). This compound, however, failed to cause any discernible change in the transport of GW274150 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 ( $K_m \sim 12 \mu\text{M}$ ) than that inferred in this study for the same amino acids from inhibition studies ( $K_i$  40–130  $\mu\text{M}$ ).  $y^+$ L is however emerging as a family of distinct heterodimeric proteins which, although characterised by their dependency on  $\text{Na}^+$  for transport of neutral amino acids and  $\text{Na}^+$ -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 ( $K_m \sim 20\text{--}30 \mu\text{M}$  vs  $K_m$  of  $\sim 340 \mu\text{M}$  for L-arginine (Pfeiffer et al., 1999)) while

$y^+$ LAT-2 shows a marginal preference for cationic substrates ( $K_m$  for L-arginine  $\sim 120 \mu\text{M}$  vs 236–295  $\mu\text{M}$  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 (Messerli 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  $\text{Na}^+$ -dependent and  $\text{Na}^+$ -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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