Uptake of T-2307, a novel arylamidine, in Candida albicans

Hiroshi Nishikawa*, Eio Yamada, Tatsuya Shibata, Shinsuke Uchihashi, Haitian Fan, Hiroyoshi Hayakawa, Nobuhiko Nomura and Junichi Mitsuyama

Research Laboratories, Toyama Chemical Co., Ltd, 2-4-1 Shimookui, Toyama, Japan

*Corresponding author. Tel: +81-76-431-8268; Fax: +81-76-431-8208; E-mail: hiroshi_nishikawa@toyama-chemical.co.jp

Methods: C. albicans cells and rat hepatocytes were exposed to 0.02 µM [14C]T-2307. After incubation, the reaction mixture was concentrated and layered on a silicon layer (mixture of silicon oil and liquid paraffin) inside a tube. The tube was then centrifuged to transfer cells into the bottom layer (sodium hydroxide) for solubilization. The bottom layer was neutralized and measured for radioactivity.

Results: T-2307 was concentrated from the extracellular medium by C. albicans cells in 10 mM phosphate buffer solution supplemented with 1% glucose by 3200- to 5100-fold. The accumulation was approximately two orders of magnitude greater than that achieved with a rat hepatocyte preparation. T-2307 uptake was sensitive to temperature and extracellular pH, and was reduced in the presence of inhibitors of mitochondrial respiration, oxidative phosphorylation and plasma membrane proton pump, and by an uncoupler. Furthermore, T-2307 uptake was concentration dependent and an Eadie–Hofstee plot suggested the involvement of two transport systems.

Conclusions: The considerably higher concentrations of T-2307 were selectively accumulated in C. albicans via transporter-mediated systems, as compared with the concentrations in rat hepatocytes. This transporter-mediated uptake of T-2307 contributes to its potent antifungal activity.

Keywords: diamidine compounds, transporter-mediated systems, potent antifungal activity

Introduction

Invasive mycoses are serious life-threatening infections in immunocompromised patients.

Antifungal drugs that are available for the treatment of these infections includeazole derivatives, candin derivatives and amphotericin B.azole antifungal agents are widely used for the treatment of fungal infections due to their broad-spectrum activities and improved safety profiles. However, there is great concern about the development ofazole-resistant isolates of Candida albicans due to their widespread, prolonged use. Candin antifungal agents show potent antifungal activity against Candida and Aspergillus; however, they show no antifungal activity against Cryptococcus neoformans. Amphotericin B exhibits broad-spectrum and fungicidal activity; however, due to the significant side effects that it causes, its clinical utility is limited to controlled intravenous administration.

Therefore, it is imperative that new antifungal agents that have potent broad-spectrum activities, novel mechanisms of action and fewer side effects are developed.

T-2307, i.e. 4-[3-[1-(3-4-(amino(imino)methyl)phenoxy)propyl]piperidin-4-yl]propoxy]benzamidine, is a novel arylamidine synthesized by Toyama Chemical Co., Ltd. Under both in vitro and in vivo conditions, T-2307 exhibits broad-spectrum activities against the majority of fungal pathogens, including Candida spp., Cryptococcus neoformans and Aspergillus fumigatus. T-2307 exhibits potent in vitro and in vivo activity, particularly against C. albicans.

T-2307 belongs to the class of aromatic diamidines that includes pentamidine and furamidine (DB75). Pentamidine is a drug that has been widely used to treat pneumocystosis, leishmaniasis and trypanosomiasis. Pafuramidine (DB289) had been developed as a prodrug of furamidine (DB75), and had been demonstrated to have good efficacy against trypanosomiasis, pneumocystosis and malaria.

It is generally accepted that the preferential uptake of these diamidine compounds by parasite-specific transporters contributes to the specific antiparasitic activities by facilitating the intracellular accumulation of these drugs to toxic concentrations. We therefore hypothesized that T-2307 is also transported into
C. albicans via a transporter-mediated system. In this report, we studied the uptake of $^{14}$C-T-2307 in C. albicans to determine whether C. albicans has a T-2307 transport system.

**Materials and methods**

**Materials**

$^{14}$C-T-2307, i.e. 4-(3-{[3-[(3-4-aminooimino)methyl]phenoxy]propyl}piperidin-4-yl)propoxy)benzyl$^{14}$C-amidine (765.77 GBq/mol), was custom synthesized by Daiichi Pure Chemicals Co., Ltd (Ibaraki, Japan). Pentamidine isethionate was obtained commercially from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). Potassium cyanide (KCN) and N,N'-dicyclohexylcarbodiimide (DCCD) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). 2,4-Dinitrophenol was purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Sodium azide, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and sodium orthovanadate were purchased from Sigma–Aldrich (St Louis, MO, USA).

**Strain and culture conditions**

C. albicans TIMM 1623 was kindly provided by Professor Hideyo Yamaguchi of Teikyo University Institute of Medical Mycology. C. albicans ATCC 90028, ATCC 10261, ATCC 64550 and ATCC MYA-574 were obtained from the American Type Culture Collection (ATCC). The cells were cultured at 35°C overnight in yeast extract/petitone/dextrose (YPD) medium. The YPD medium consisted of 1% Bacto yeast extract (Becton, Dickinson and Company, Sparks, MD, USA), 2% Bacto peptone (Becton, Dickinson and Company) and 2% glucose. The cells were harvested during the log phase of growth by centrifugation at 1700 g for 10 min at room temperature. After washing with saline, the cells were suspended in 10 mM phosphate buffer solution (PB) or 10 mM PB supplemented with 1% glucose (PBG).

**Isolation of rat hepatocytes**

Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbitol (50 mg/kg; Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) and subjected to celiotomy. The liver was perfused for 5 min with liver perfusion medium (Invitrogen Corporation, Carlsbad, CA, USA) warmed to 39°C (at 15 mL/min along with oxygen) and then perfused for 10–15 min with liver digest medium (Invitrogen Corporation) warmed to 39°C (at 20 mL/min along with oxygen). After perfusion, the liver sample was transferred into a cell culture dish filled with 20 mL of ice-cold William’s medium E (Sigma–Aldrich). Cellulor aggregates and tissue debris were removed, and the hepatocytes were gently suspended using a disposable pipette. The hepatocyte suspension was filtered using a cell strainer on ice and centrifuged at 50 g for 3 min at 4°C to collect hepatocytes. The hepatocytes were then washed with 30 mL of hepatocyte wash medium (Invitrogen Corporation) and were gently suspended with 15–20 mL of William’s medium E. The hepatocyte suspension was mixed with 10× Hank’s buffered salt solution and Percoll (Sigma–Aldrich) (mixture ratio, 10:1:9, v/v/v), and centrifuged at 50 g for 10 min at 4°C. The supernatant containing dead cells was removed and the pellet was gently suspended with a small quantity of William’s medium E. The cells were then counted to estimate their viability after staining with Trypan Blue. Subsequently, the hepatocyte suspension was diluted with William’s medium E supplemented with 5 mM glucose. All animal experimental procedures were conducted in accordance with the guidelines for care and use of laboratory animals at Toyama Chemical Co., Ltd.

**Kinetic studies of uptake**

The apparent kinetic parameters, $K_m$ (Michaelis constant) and $V_{max}$ (maximum transport rate), of T-2307 uptake in C. albicans were calculated by non-linear least-squares regression analysis using WinNonlin software (Pharsight Inc., Mountain View, CA, USA), according to the following Michaelis–Menten-type equations:

$$V = V_{max}[S]/(K_m + [S])$$  \hfill (1)

$$V = V_{max1}[S]/(K_{m1} + [S]) + V_{max2}[S]/(K_{m2} + [S])$$  \hfill (2)

where $V$ and [S] are the velocity of substrate uptake and saturable concentration, respectively. Data were fitted to Equation (2) with two transport components, where the indices 1 and 2 indicate the high- and low-affinity components, respectively.

**Results**

$^{14}$C-T-2307 uptake in C. albicans cells and rat hepatocytes

C. albicans cells were exposed to 0.02 μM $^{14}$C-T-2307 for 30 min at 30°C in PB and PBG (pH 7.4) (Table 1). We set the starting
extracellular concentration of [14C]T-2307 to a low level (0.02 μM) based on the MIC against C. albicans and the detection limit of radioactivity, T-2307 has no impact on cell viability in the time frame examined, which could have influenced the uptake. C. albicans TIMM 1623 cells accumulated [14C]T-2307 up to a concentration of 27.33 ± 1.07 and 57.54 ± 2.71 pmol/10^7 cells after incubation in PB and PBG, respectively. The addition of glucose resulted in a 2-fold increase in the uptake of [14C]T-2307 in C. albicans TIMM 1623, indicating that T-2307 uptake was energy dependent. T-2307 was also highly accumulated in azole-resistant C. albicans ATCC 64550 and ATCC MYA-574 overexpressing the ABC transporter genes CDR1 and CDR2, which encode ATP-dependent efflux pumps, suggesting that T-2307 was not effluxed by these pumps.

The intracellular concentrations in C. albicans TIMM 1623 cells in PB and PBG were 43.7 and 92.1 μM, respectively, which were ~2200- to 4600-fold higher than the starting extracellular concentration. The intracellular concentrations in other C. albicans cells were ~3200- to 5100-fold higher than the starting extracellular concentration. On the other hand, the intracellular concentration in rat hepatocytes was 0.691 μM after incubation with 0.02 μM [14C]T-2307 for 30 min at 37°C in William’s medium E supplemented with 5 mM glucose.

Next, to study the effect of extracellular pH on T-2307 uptake, the uptake of 0.02 μM [14C]T-2307 in C. albicans TIMM 1623 was measured in PBG (pH 5.0–8.0) at 30°C for 5 min (Figure 1b). The pH dependence data showed decreasing uptake with decreasing pH. The data were not consistent with the idea that the cationic species T-2307 simply equilibrates across the plasma membrane in response to the pH gradient or the idea that proton symport plays a role in T-2307 uptake. These data suggest that the protonation state of key amino acid residues within the T-2307 transporter would affect the uptake.

### Effects of temperature and extracellular pH on [14C]T-2307 uptake in C. albicans

To determine whether T-2307 uptake is sensitive to temperature, the uptake of 0.02 μM [14C]T-2307 in C. albicans TIMM 1623 was measured in PBG (pH 7.4) at 30°C and 4°C (Figure 1a). The uptake at 30°C was time dependent and linear for 5 min. Simultaneous controls incubated at 4°C exhibited very low uptake of [14C]T-2307. After incubation for 5 min, C. albicans accumulated [14C]T-2307 up to a concentration of 33.58 ± 0.89 μM at 30°C and 3.98 ± 0.47 μM at 4°C. The extent of the temperature sensitivity was indicative of a transporter-mediated mechanism of uptake, rather than diffusion.
the actively metabolizing mitochondrion, with uptake being driven by the mitochondrial membrane potential.

Organisms that make use of active transport by proton motive force generally have a DCCD-sensitive proton-extruding ATPase on their plasma membrane, which maintains the transmembrane proton and electrical gradients. DCCD (100 µM) significantly decreased T-2307 uptake (38.55%±3.19% of the control). Sodium orthovanadate (5 mM), a plasma membrane-type proton ATPase inhibitor, also significantly reduced T-2307 uptake (60.51%±6.29% of the control). These results suggested that the plasma membrane pH gradient would play a role in T-2307 uptake. However, these results were not consistent with the pH dependence results.

DCCD, a non-specific proton ATPase inhibitor, inhibits not only plasma membrane proton ATPase but also mitochondrial proton ATPase. Vanadate is also known to affect mitochondrial respiration by altering electron transfer between complexes III and IV. In view of the findings and the pH dependence data, these results indicated that T-2307 uptake would be affected by the intracellular pH or mitochondrial metabolism rather than by the plasma membrane pH gradient.

**[^14C]T-2307 uptake in C. albicans in the presence of different quantities of unlabelled T-2307 and pentamidine**

To further characterize T-2307 uptake in C. albicans, the uptake of 0.02 µM[^14C]T-2307 uptake in C. albicans TIMM 1623 was measured for 5 min in the presence of various concentrations of unlabelled T-2307 (0.01 – 100 µM). T-2307 uptake was concentration dependent (Figure 2a and b) and an Eadie–Hofstee plot clearly yielded two slopes (Figure 2c and d). This indicated the involvement of two different transport systems. We obtained the velocity by subtraction of the uptake at 4°C as the binding to the cell wall and the non-specific uptake from the uptake at 30°C, indicating that neither of the two transport systems represented the binding and the non-specific uptake. Therefore, we fitted the data to a two-transporter model and determined the apparent kinetic parameters for two transport systems. The apparent kinetic parameters were as follows: high-affinity transport system (K<sub>m1</sub> = 0.0234±0.0092 µM, V<sub>max1</sub> = 0.159±0.024 pmol/10<sup>7</sup> cells/s) and low-affinity transport system (K<sub>m2</sub> = 102±26 µM, V<sub>max2</sub> = 50.4±9.5 pmol/10<sup>7</sup> cells/s).

We already showed that the uptake of T-2307 at low concentration (0.02 µM) was temperature and pH sensitive, and energy dependent. Furthermore, in the presence of 100 µM unlabelled T-2307, the uptake of 0.02 µM[^14C]T-2307 was reduced by ~94% (Figure 3), indicating that the majority of T-2307 uptake at low concentration occurs via a saturable, carrier-mediated process, with only minor non-saturable component. These data indicated that the high-affinity transport system could be a carrier-mediated system.

To determine whether the additional low-affinity transport system is also a carrier-mediated system, T-2307 uptake in C. albicans TIMM 1623 at a higher concentration was studied. After incubation with 100 µM T-2307 for 5 min at 30°C, C. albicans cells accumulated T-2307 up to a concentration of 18.42±0.75 mM in PBG and 5.42±0.34 mM in PB. Under the same conditions, sodium azide (5 mM) and 2,4-dinitrophenol...
(5 mM) significantly decreased the T-2307 uptake in PBG (19.61% ± 2.48% and 59.23% ± 5.44% of the control, respectively). After incubation with 100 μM T-2307 for 5 min at 4°C in PBG, C. albicans cells accumulated T-2307 up to a concentration of 6.91 ± 0.43 mM. These results suggested that the low-affinity transport system was temperature sensitive and energy dependent.

Because the structure of T-2307 is similar to that of diamidines such as pentamidine, we hypothesized that a common transport system is responsible for the accumulation of these compounds in C. albicans. To test this hypothesis, the uptake of 0.02 μM [14C]T-2307 in C. albicans TIMM 1623 was measured in the presence of various concentrations of unlabelled T-2307 (0.01–100 μM) at 30°C for 5 min in PBG (pH 7.4). We used a 5 min timepoint, during which uptake was linear (see Figure 1a), to determine the kinetic constants for uptake. These results were obtained by subtraction of the T-2307 uptake at 4°C as the non-specific uptake from the uptake at 30°C. The results are expressed as means ± SEM of triplicate experiments. (b) T-2307 uptake in C. albicans TIMM 1623 at low concentration in (a). (c) Eadie–Hofstee plot of the T-2307 uptake in C. albicans TIMM 1623. S, T-2307 concentration (μM); V, initial rate of T-2307 uptake (pmol/10^7 cells/s). (d) Eadie–Hofstee plot of the T-2307 uptake in C. albicans TIMM 1623 at low concentration in (c).

Discussion
In the present study, we characterized the transport properties of the novel arylamidine antifungal agent T-2307 across the cell membrane of C. albicans. These data indicated that T-2307 was transported into C. albicans via two transport systems. Based on the determination of apparent K_m and V_max values for these transport systems, it is predicted that ~90% of the T-2307 uptake via the transport systems is mediated via the high-affinity carrier at the MIC against C. albicans (0.001 mg/L, ~0.002 μM). We consider that the typical potent antifungal activity of T-2307 arises due to the high accumulation via the high-affinity carrier.
Pentamidine was found to be an inhibitor of arginine transport. However, a previous study showed that pentamidine is saturable high-affinity transporter. The presence of the required to give significant counts of accumulated [14C]T-2307 could represent uptake into the mitochondrion. Thus, the second non-saturable component was measured in the time period tested, although the T-2307 uptake study at higher concentration suggested that it could be a low-affinity carrier.

Figure 3. Uptake of [14C]T-2307 in C. albicans in the presence of various concentrations of unlabelled T-2307 and pentamidine. Uptake of 0.02 mM [14C]T-2307 in C. albicans TIMM 1623 was measured in the presence of various concentrations of unlabelled T-2307 and pentamidine (0.01–100 µM) at 30°C for 5 min in PBG (pH 7.4). These results were obtained by subtraction of the T-2307 uptake at 4°C as the non-specific uptake from the uptake at 30°C. The results are expressed as means ± SEM of triplicate experiments.

In African trypanosomes, pentamidine uptake appears to involve the P2 nucleoside transporter and two additional transporters, a low-capacity high-affinity pentamidine transporter (HAPT1) and a high-capacity low-affinity pentamidine transporter (LAPT1). Loss of the P2 nucleoside transporter can mediate resistance to diamidines. The P2 nucleoside transporter displayed high affinity (K_u = 0.43 ± 0.02 µM) for pentamidine. The KM values for pentamidine transport into Trypanosoma brucei via HAPT1 and LAPT1 were 0.036 ± 0.006 and 56.2 ± 8.3 µM, respectively. These KM values were comparable to the apparent KM values for the T-2307 uptake.

In Leishmania spp., pentamidine uptake has been shown to be saturable, involving a carrier-mediated, energy-dependent process. However, a previous study showed that pentamidine did not enter Leishmania parasites via a nucleoside transporter. Pentamidine was found to be an inhibitor of arginine and polyamine transport in Leishmania donovani and Leishmania mexicana. However, experiments using radiolabelled pentamidine in L. mexicana failed to show reciprocal inhibition with arginine and polyamines.

Pentamidine uptake in L. donovani has also been reported to occur via a secondary non-saturable route in addition to the saturable high-affinity transporter. The presence of the second route was indicated by a failure of up to 1 mM cold pentamidine to completely inhibit the uptake of 1 µM [3H]pentamidine. Furthermore, the secondary route could be measured in wild-type cells but not in resistant cells with decreased mitochondrial membrane potential. The authors speculated that fast accumulation of pentamidine into the mitochondrion removed the drug from the cytosol and maintained a concentration gradient across the plasma membrane, allowing continuous uptake in cells; thus, the second non-saturable component could represent uptake into the mitochondrion.

In the kinetic study of T-2307, a 5 min uptake period was required to give significant counts of accumulated [14C]T-2307 and this appeared to fall within the linear range (Figure 1a). Considering that diamidines are accumulated into the mitochondrion within a few minutes, it cannot be ruled out that this time period actually represents not only initial uptake rates across the plasma membrane but also the accumulation into the mitochondrion. The inhibitor study indicated that T-2307 would be accumulated into the mitochondrion within this time period, with uptake being driven by the mitochondrial membrane potential. Furthermore, the inhibitory effect of unlabelled T-2307 to 0.02 µM [14C]T-2307 uptake became weaker at a concentration of >1 µM and even 100 µM unlabelled T-2307 failed to completely inhibit the [14C]T-2307 uptake (Figure 3). In view of these results and the report about pentamidine, the low-affinity component indicated by the Eadie–Hofstee plot could represent T-2307 accumulation into the mitochondrion within the time period tested, although the T-2307 uptake study at higher concentration suggested that it could be a low-affinity carrier.

The uptake of [14C]T-2307 in C. albicans was inhibited by pentamidine only at higher concentrations than unlabelled T-2307, suggesting that pentamidine has lower affinity to the T-2307 transport system. On the other hand, it cannot be ruled out that pentamidine inhibits the function required for T-2307 uptake at higher concentrations; although it has been reported that 100 µM pentamidine has almost no inhibitory effect on the respiration of whole yeast cells. Further studies are required to determine the inhibitory behaviour of pentamidine on T-2307 uptake and to identify the T-2307 transporter.

Lanteri et al. reported that Saccharomyces cerevisiae cells grown in a medium containing glycerol, which is the non-fermentative carbon source, were more sensitive to the growth-inhibitory effects of DB75 or pentamidine than cells grown in a medium containing dextrose, suggesting that DB75 and pentamidine inhibit the mitochondrial functions of cells. Similarly, in a study of T-2307, S. cerevisiae cells cultured in a medium containing glycerol were more susceptible to the growth-inhibitory effects of T-2307 than those cultured in a medium containing glucose. Therefore, we now consider that one of the mechanisms of action of T-2307 may be similar to that of DB75 and pentamidine.

In conclusion, the considerably higher concentrations of T-2307 were selectively accumulated in C. albicans via transporter-mediated systems, as compared with the concentrations in rat hepatocytes. This transporter-mediated uptake of T-2307 in C. albicans contributes to its potent antifungal activity.

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All of the authors are employed by Toyama Chemical Co., Ltd. None of the authors owns stock or options in the company.
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