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ORIGINAL ARTICLE

Testicular disposition of clofarabine in rats is dependent on equilibrative nucleoside transporters

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Abstract

Acute lymphoblastic leukemia (ALL) is the most common cancer in children and adolescents. Although the 5-year survival rate is high, some patients respond poorly to chemotherapy or have recurrence in locations such as the testis. The blood–testis barrier (BTB) can prevent complete eradication by limiting chemotherapeutic access and lead to testicular relapse unless a chemotherapeutic is a substrate of drug transporters present at this barrier. Equilibrative nucleoside transporter (ENT) 1 and ENT2 facilitate the movement of substrates across the BTB. Clofarabine is a nucleoside analog used to treat relapsed or refractory ALL. This study investigated the role of ENTs in the testicular disposition of clofarabine. Pharmacological inhibition of the ENTs by 6-nitrobenzylthioinosine (NBMPR) was used to determine ENT contribution to clofarabine transport in primary rat Sertoli cells, in human Sertoli cells, and across the rat BTB. The presence of NBMPR decreased clofarabine uptake by 40% in primary rat Sertoli cells ($p = .0329$) and by 53% in a human Sertoli cell line ($p = .0899$). Rats treated with 10 mg/kg intraperitoneal (IP) injection of the NBMPR prodrug, 6-nitrobenzylthioinosine 5′-monophosphate (NBMPR-P), or vehicle, followed by an intravenous (IV) bolus 10 mg/kg dose of clofarabine, showed a trend toward a lower testis concentration of clofarabine than vehicle (1.81 ± 0.59 vs. 2.65 ± 0.92 ng/mg tissue; $p = .1160$). This suggests that ENTs could be important for clofarabine disposition. Clofarabine may be capable of crossing the human BTB, and its potential use as a first-line treatment to avoid testicular relapse should be considered.

KEYWORDS
blood-testis barrier, clofarabine, nucleoside transporter, nucleosides, Sertoli cells

Abbreviations: ALL, Acute lymphoblastic leukemia; BCRP, Breast cancer resistance protein; BTB, Blood-testis barrier; CNT, Concentrative nucleoside transporter; ENT, Equilibrative nucleoside transporter; HIV, human immunodeficiency virus; NBMPR, S-[(4-nitrophenyl)methyl]-6-thioinosine; NRTI, nucleoside reverse transcriptase inhibitor; OAT, Organic anion transporter; OCT, Organic cation transporter.

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1 | INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common cancer in children and adolescents, and more than 3000 new cases are diagnosed yearly.[1,2] Improved treatment of ALL has increased the 5-year survival rate to 82%.[2] However, there are ALL patients who do not respond to chemotherapy or have a cancer recurrence after treatment, with the most common relapses occurring in the bone marrow, central nervous system, or gonads.[3–10] Acute myeloid leukemia (AML) is a common cancer among adults, with a much lower 5-year survival rate of 29%.[11–13] Sanctuary sites such as the central nervous system or gonads may provide a location where the cancer can evade first-line treatments.[3–7,9] Testicular relapse of ALL or AML may require orchiectomy (complete removal of one or both testes) or irradiation, leaving the patient with reduced fertility or irreversible infertility,[8] which can have a devastating quality of life impact. The testis is an immune privileged site, which prevents autoimmunity against germ cells, but impedes the immune system response to cancer present within the testis.[14,15]

Sanctuary sites can hinder complete eradication of viral infections including Ebola virus and human immunodeficiency virus (HIV).[16,17] Without treatment, these viruses can be sexually transmitted. Newly developed monoclonal antibodies are being used to treat Ebola virus,[18,19] and antivirals including nucleoside reverse transcriptase inhibitors (NRTIs) and HIV protease inhibitors have a long history of success in treating HIV.[20]

Developing germ cells are protected by the blood–testis barrier (BTB), which is composed of physical and physiological components. This includes the tight junctions present between adjacent Sertoli cells and efflux transporters present at the basal membranes of Sertoli cells.[21–23] Transporters are one of the most effective ways for drugs to cross the BTB. Drugs can circumvent the BTB through transepithelial transport pathways present in Sertoli cells.[24] Chemotherapies that are substrates for efflux transporters present at the BTB may lead to inadequate treatment of cancer and facilitate relapse.

Clofarabine is a nucleoside analog used to treat pediatric patients with relapsed or refractory ALL and is successful in treating AML.[25–27] Clofarabine is converted intracellularly to its active phosphorylated form to inhibit DNA synthesis.[26,28] Clofarabine was determined to be a substrate of the organic anion transporters 1 and 3 (OAT1 and OAT3) and the organic cation transporters 1 and 2 (OCT1 and OCT2).[26] Additional studies demonstrated that clofarabine is a substrate for breast cancer resistance protein (BCRP), concentrative nucleoside transporters 1 and 3 (CNT1 and CNT3), and the equilibrative nucleoside transporters 1 and 2 (ENT1 and ENT2).[29–31] mRNA expression of CNTs is detectable in Sertoli cells at low levels, but little is known about protein expression or localization of these transporters at the BTB.[32,33] The transepithelial transport pathway created by ENT1 on the basal membrane of Sertoli cells and ENT2 on the apical membrane allows for nucleoside analog entry into the adluminal compartment, where they can reach therapeutic concentrations within the male genital tract.[34]

Lamivudine is an NRTI used to treat HIV-1 infection.[35,36] It is detectable in the semen of patients prescribed lamivudine, indicating that it is capable of crossing the BTB.[37–39] Lamivudine is a well-established substrate of the OCTs [40,41] and did not interact with ENTs in previous studies.[31,42]

The current study evaluated the impact of ENTs in the disposition of clofarabine and lamivudine to the testis. ENT-dependent drug uptake can be studied with the use of an ENT specific inhibitor, S-[(4-nitrophenyl)methyl]-6-thioinosine (NBMPR). Nanomolar concentrations of NBMPR inhibit ENT1, whereas micromolar concentrations inhibit ENT2.[31,33,34,42–44] 6-Nitrobenzylthioinosine 5′-monophosphate (NBMPR-P) is the more soluble prodrug of NBMPR that is rapidly converted to NBMPR in vivo[45] and was used to pharmacologically inhibit the ENTs in rats. Primary rat Sertoli cells and a human Sertoli cell line were used to determine if clofarabine and lamivudine enter Sertoli cells through an ENT-dependent mechanism. The data presented suggest that clofarabine crosses the BTB in rodents through the ENTs and enters human Sertoli cells through these transporters and that lamivudine is capable of crossing the BTB in an ENT-independent manner. Other nucleoside analogs may cross the BTB using the ENT1–ENT2 transepithelial transport pathway. This may explain the effectiveness of clofarabine in reaching this sanctuary site for the treatment of refractory or relapsed leukemias. Additionally, the ENTs are expressed in leukemia cells, and lower ENT expression levels are linked to drug resistance.[46–50] A first-line treatment that is capable of reaching the testis could be used to avoid testicular relapse and eliminate the need for radiation therapy or orchiectomy rendering patients with reduced fertility or infertile.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

NBMPR-P was purchased from Toronto Research Chemicals. NBMPR was purchased from Tocris Bioscience. Clofarabine, cladribine, and lamivudine were purchased from Cayman Chemical. Sterile 1X Phosphate Buffered Saline was purchased from Research Products International. Heparin was purchased from Alfa Aesar. Poly-L-lysine was purchased from Sciencell. PEG-400 was purchased from Sigma-Aldrich. Collagenase type 1 was purchased from Worthington Biochemical Corporation. Additional reagents were purchased from Thermo Fisher Scientific unless otherwise noted.

2.2 | hTERT-immortalized human Sertoli cell culture

An immortalized human Sertoli cell line generated from primary human Sertoli cells overexpressing human telomerase reverse transcriptase (hTERT) was cultured as previously described by our laboratory.[33] Cells were grown in dulbecco's modified eagle medium (DMEM)/F12 (Sigma-Aldrich) supplemented with 10 μg/ml human insulin, 2.5 ng/ml epidermal growth factor, 10% fetal bovine serum, and 1% penicillin and streptomycin.[33] Passages 33–35 were used for

uptake experiments. Cells were seeded (15,000 cells/well) on human fibronectin-coated 96 well plates (Corning Inc).

2.3 | Animals

Procedures involving live rats were approved by the University of Arizona Institutional Animal Care and Use Committee. Nine-week-old Sprague–Dawley rats were purchased from Charles River Laboratories and housed in 12-h on/off light cycles with three rats per cage. Rats were given water and standard chow ad libitum. Cell isolation for in vitro studies and separate in vivo studies were completed after rats underwent a 1-week acclimatization period. Rats were euthanized by carbon dioxide asphyxiation, and death was confirmed with diaphragm puncture.

2.4 | Primary rat Sertoli cell isolation and culture

Sertoli cells were isolated by a previously established method from three different naïve 10-week-old Sprague–Dawley rats.[51] Briefly, rats were euthanized, and testicles were removed and placed in sterile Dulbecco's modified phosphate buffered saline (DPBS). Testicles were detunicated, minced, and resuspended in minimal media (DMEM/F12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) before centrifugation. Supernatant was discarded with each wash. This process was repeated three times to partially clean up tissue debris. Minimal media containing 2 mg/ml collagenase, 1 μg/ml soybean trypsin inhibitor, 10 μg/ml DNase, and 1 mg/ml hyaluronidase was added to dissociate and digest the tissue extracellular matrix for 30 min at 32°C with gentle agitation. The digested tissues were centrifuged for 10 min at 100 *g* at 20°C, washed three times with minimal media, and passed through a 70-μM nylon mesh filter. The cells were plated on a 2 μg/cm^2 poly-L-lysine-coated cell culture flask and grown in minimal media supplemented with 10 μg/ml human insulin and 2.5 ng/ml epidermal growth factor in a 35°C humidified 5% CO_2 incubator.

Isolated cells underwent a hypotonic treatment to remove contaminant germ cells 24 h after isolation. The old media was aspirated before adding DPBS (pH 7.4) supplemented with 20-mM Tris into the flasks for 5 min and then replacing with supplemented media.[52] Cells were passed as previously described and passed one time before seeding for experiments.[53] The second passage after isolation (i.e., first passage after hypotonic treatment) was used for transport experiments. Cells from three different rats/cell isolations were used. For drug uptake experiments, cells were seeded (15,000 cells/well) on human fibronectin-coated 96 well plates (Corning Inc).

2.5 | Drug uptake into primary rat Sertoli cells and hTERT-immortalized human Sertoli cells

Experiments were completed with confluent monolayers of cells and completed as previously described.[31] For clofarabine experiments, cells were washed once with 200 μl of Waymouth's buffer (WB) before adding 50 μl of WB containing 50-μM clofarabine with or without NBMPR (100 μM). For lamivudine experiments, cells were washed once with 200 μl of WB before adding 50 μl of WB containing 200-μM lamivudine with or without NBMPR (100 μM). These concentrations of NBMPR completely inhibit ENT1 and ENT2.[31,43,54] Transport experiments were terminated after 5 min, and cells were rinsed twice with 200 μl of WB. Fifty microliters of 1:1 methanol (MeOH):acetonitrile (ACN) was added to cells containing 200 ng/ml of internal standard (cladribine) and incubated overnight at 4°C. The calibration curves were prepared in MeOH:ACN and treated identically to samples (0–250 ng/ml). Samples were dried and resuspended the following day in 50 μl of H_2O + 0.1% formic acid for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

2.6 | Testicular disposition of clofarabine and lamivudine

Clofarabine was dissolved in 100% v/v PEG-400 at a concentration of 10 mg/ml and subsequently diluted 1:1 in sterile saline for a final concentration of 5 mg/ml. Lamivudine was prepared at a concentration of 10 mg/ml in sterile saline. NBMPR-P was dissolved in 100% v/v DMSO at 50 mg/ml, diluted to 25 mg/ml in 75% v/v DMSO in sterile saline, and diluted to a final concentration of 2.5 mg/ml in sterile saline (final DMSO concentration 7.5% v/v). Vehicle was prepared as 7.5% v/v DMSO in sterile saline. Solutions were sterilized by passage through a 0.2-μm filter. The contents were removed aseptically with a sterile needle and syringe. The top of the septum was disinfected with 70% v/v ethanol prior to use.

Animals were divided into two groups per drug: one receiving NBMPR-P and one receiving vehicle (7.5% DMSO in sterile saline). Previous studies indicated that a dose of 15 mg/kg of NBMPR-P in mice resulted in a maximum plasma concentration of NBMPR after 20 min and rapidly declined with a half-life of approximately 24 min.[45] Other studies that examined clofarabine pharmacokinetics used 25 or 50 mg/kg clofarabine. Our preliminary studies indicated that a dose of 10 mg/kg was adequate to detect clofarabine in the testis, and 10 mg/kg lamivudine was an adequate dose to detect in the testis. Single intraperitoneal (IV) doses of clofarabine and lamivudine were used to minimize the complexity that arises from other routes of administration, and clofarabine is administered IV clinically. Preliminary studies also demonstrated that 10 mg/kg NBMPR-P was sufficient to reach plasma concentrations adequate to inhibit both ENT1 and ENT2 (low micromolar range). Single dose pharmacokinetics of NBMPR after NBMPR-P intraperitoneal (IP) administration was described in Gati et al. and produced an adequate concentration to inhibit both ENT1 and ENT2.[45]

Animals received 10 mg/kg of NBMPR-P or vehicle by intraperitoneal injection 20 min prior to receiving drug at 10 mg/kg via intravenous bolus dose. Ten minutes after receiving drug, animals were euthanized, and testes were collected. Testicles were weighed,

detunicated, snap frozen in liquid nitrogen, and stored at −80°C for later analysis.

2.7 | LC-MS/MS quantification of clofarabine, lamivudine and NBMPR

Clofarabine, lamivudine, and NBMPR concentrations were quantified in rat plasma using LC-MS/MS. Briefly, 400 µl of acetonitrile containing 200 ng/ml of internal standard (cladribine) was added to 10 µl of plasma and centrifuged at maximum speed (15,000 *g*) for 15 min at 4°C. Three hundred microliters of supernatant was collected and dried in a vacuum centrifuge. Samples were resuspended in 100 µl of 90:10 H_2O:ACN +0.1% formic acid for NBMPR quantification and 100 µl of H_2O + 0.1% formic acid for clofarabine and lamivudine quantification. Calibration curves were prepared identically to samples using naïve rat plasma and spiking in compound. The plasma calibration ranges were 937.5–15,000 ng/ml.

Clofarabine and lamivudine concentrations in the testis were quantified using LC-MS/MS. Briefly, approximately 100 mg of tissue was weighed out and suspended in 1-ml/100-mg tissue of 80:20 methanol:H_2O containing 62.5 ng/ml internal standard (cladribine). Tissues were homogenized with a rotary tissue grinder for 20 s. Approximately 300 µl of homogenate was centrifuged at 15,000 *g* for 15 min at 4°C. Two hundred microliters of supernatant was collected and dried into a pellet using a vacuum centrifuge. Samples were resuspended in 100 µl of H_2O + 0.1% formic acid for clofarabine quantification. Calibration curves were prepared identically to samples using naïve rat testis and spiking each sample with clofarabine. The testis calibration ranges for clofarabine and lamivudine were 62.5–1000 ng/ml.

A Shimadzu LC system (Shimadzu) equipped with a 3.0 × 50 mm, 2.7-µm particle InfinityLab Poroshell 120 EC-C18 column (Agilent) was used to chromatograph 10 µl of each sample with a binary gradient composed of 0.1% formic acid in H_2O (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). All analytes were detected using multiple reaction monitoring (MRM) by a Sciex® QTrap 450 (Sciex) operated in positive electrospray ionization. The internal standard, cladribine, was measured in each method.

For NBMPR, with a flow rate of 0.3 ml/min, the following gradient was used over 6 min: 1 min, 10% B; 3 min, 90% B; 4 min, 90% B; and 4.5 min, 10% B. The column was equilibrated for 1.5 min at 10% B between samples. The following parameters were used for detection:

Compound	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)
NBMPR	420.1	288.0	101	25
Cladribine (IS)	286.0	170.0	30	25

For clofarabine, with a flow rate of 0.250 ml/min, the following gradient was used over 5.1 min: 1 min, 0% B; 5 min, 80% B; 6 min, 80% B; and 7 min, 0% B. The column was equilibrated for 2 min at 0% B between samples. The following parameters were used for detection:

Compound	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)
Clofarabine	304.1	170.1	50	28
Cladribine (IS)	286.0	170.0	30	25

For lamivudine, with a flow rate of 0.4 ml/min, the following gradient was used over 5.1 min: 1 min, 2.5% B; 4 min, 90% B; 4.5 min, 90% B; and 5 min, 2.5% B. The column was equilibrated for 2 min at 2.5% B between samples. The following parameters were used for detection:

Compound	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)
Lamivudine	230.0	112.0	85	35
Cladribine (IS)	286.0	170.0	30	25

All LC-MS/MS data were analyzed using MultiQuant MD version 3.0.2 before performing statistical analysis using cladribine as an internal standard (IS) for quantification. In vitro experimental data were converted from ng/ml to pmol/cm^2 before normalizing to uptake in the absence of NBMPR, plasma concentrations were converted from ng/ml to µM, and tissue concentrations were converted to ng drug per mg tissue.

2.8 | Data analysis

All data were analyzed using GraphPad Prism 8. An unpaired two-tailed *t*-test was used to identify a decrease in clofarabine uptake experiments in Sertoli cells and clofarabine testis concentrations between groups ($p \leq .05$ indicating a difference), although previous data suggest clofarabine is a substrate of the ENTs, and clofarabine uptake was decreased in the presence of NBMPR in ENT1 and ENT2 cells.[31,55] An unpaired two-tailed *t*-test was used to analyze lamivudine experiments. Data are presented as mean ± SD.

2.9 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[56] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[57]

3 | RESULTS

3.1 | Drug uptake in TERT immortalized human Sertoli cells

Clofarabine uptake was measured in the presence of 100-µM NBMPR in TERT immortalized human Sertoli cells (Figure 1A). A 53% decrease in clofarabine uptake in the presence of 100-µM NBMPR after 5 min was observed ($p = .0899$). Lamivudine uptake

was measured in the presence of 100-μM NBMPR in TERT immortalized human Sertoli cells (Figure 1B). No decrease in lamivudine uptake in the presence of 100-μM NBMPR after 5 min was observed ($p = .7276$).

3.2 | Drug uptake in primary rat Sertoli cells

Clofarabine uptake was measured in the presence of 100-μM NBMPR in isolated primary rat Sertoli cells (Figure 2A). A 40% decrease in clofarabine uptake in the presence of 100-μM NBMPR after 5 min was observed ($p = .0329$). Lamivudine uptake was measured in the presence of 100-μM NBMPR in isolated primary rat Sertoli cells (Figure 2B). No decrease in lamivudine uptake in the presence of 100-μM NBMPR after 5 min was observed ($p = .1216$).

3.3 | Testicular disposition of clofarabine

The impact of NBMPR on the reduction of clofarabine disposition to the testis was determined. The clofarabine + vehicle group had an $n = 6$, whereas the clofarabine + NBMPR-P group had an $n = 5$. One rat was excluded from the clofarabine + NBMPR-P group because the IP injection failed to deliver an adequate plasma concentration of NBMPR (i.e., NBMPR concentrations were below the limit of detection). In the clofarabine + NBMPR-P, the terminal plasma concentration of NBMPR was 9.58 ± 3.06 μM (Table 1). The clofarabine concentration in the testis was slightly lower in the clofarabine + NBMPR-P group than clofarabine + vehicle group (1.81 ± 0.59 vs. 2.65 ± 0.92 ng/mg tissue; $p = .1160$; Figure 3A); however, statistical significance was not achieved. Clofarabine plasma concentrations were not different between vehicle and NBMPR-P groups (11.12 ± 2.26 vs. 11.43 ± 1.04 μM, $p = .7840$).

3.4 | Testicular disposition of lamivudine

The impact of NBMPR on lamivudine disposition to the testis was determined. The lamivudine + vehicle group also had an $n = 6$, whereas the lamivudine + NBMPR-P group had an $n = 5$. One rat was excluded from the lamivudine + NBMPR-P group because the IP injection failed to deliver an adequate plasma concentration of NBMPR (i.e., NBMPR concentrations were below the limit of detection). In the lamivudine + NBMPR-P group, the terminal plasma concentration of NBMPR was 7.59 ± 4.08 μM (Table 1). The lamivudine concentration in the testis was not different in the lamivudine + NBMPR-P group than lamivudine + vehicle group (1.48 ± 0.11 vs. 1.58 ± 0.24 ng/mg tissue; $p = .4490$; Figure 3B). Lamivudine plasma concentrations were different between vehicle and NBMPR-P groups (25.82 ± 4.04 vs. 35.41 ± 6.74 μM, $p = .0168$).

4 | DISCUSSION

This study was the first to use pharmacological inhibition of the ENTs in Sertoli cells and in whole animals to evaluate clofarabine transport at the BTB. The data presented in this study suggest that ENTs play a role in clofarabine transport, but not lamivudine transport, in the testis. It is worth emphasizing that based on these data, lamivudine is capable of entering Sertoli cells in vitro and reaching testicular tissue in vivo, just not through an ENT-dependent mechanism. A graphical representation of clofarabine transport by the ENTs is presented in Figure 4. Previous studies demonstrate that NBMPR inhibits ENT-mediated transport in HeLa cells, PK15 cells, xenopus oocytes injected with RNA transcripts for ENTs, and Sertoli cells.[31,42,43,58] The pharmacokinetics of NBMPR after NBMPR-P administration in rodents has been characterized, and other studies used pharmacological inhibition of ENTs to investigate the impacts drug distribution.[45,59]

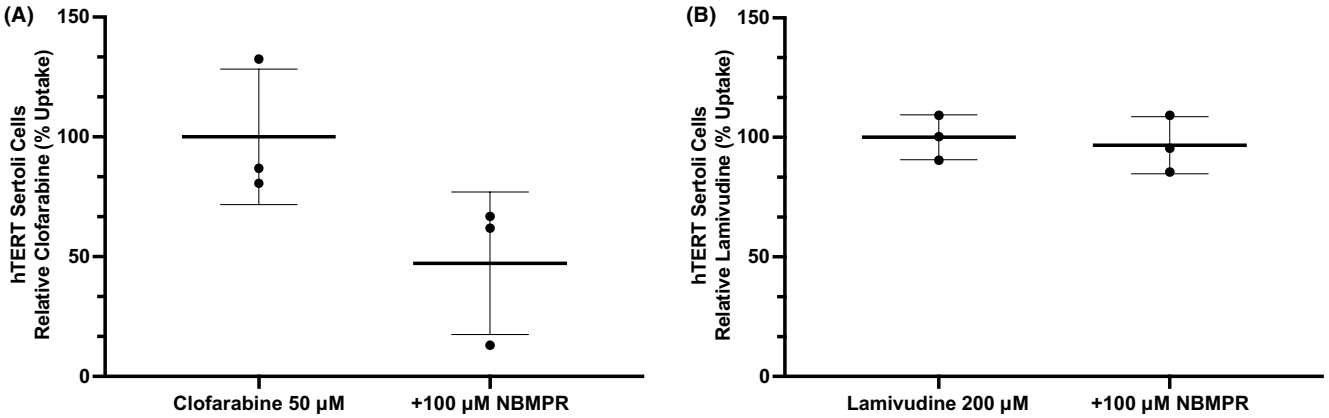

FIGURE 1 Clofarabine (A) and lamivudine (B) uptake in hTERT immortalized human Sertoli cells. Cells were incubated with 50-μM clofarabine for 5 min in the presence or absence of 100-μM NBMPR or 200-μM lamivudine in the presence or absence of 100-μM NBMPR. Data are presented as mean ± SD. Each experiment was completed in duplicate wells with $n = 3$. Each n represents cell passages 33–35 for each experiment. An unpaired, two-tailed t-test ($p \leq .05$) was used to determine statistical differences between groups. hTERT, human telomerase reverse transcriptase; NBMPR, 6-nitrobenzylthioinosine

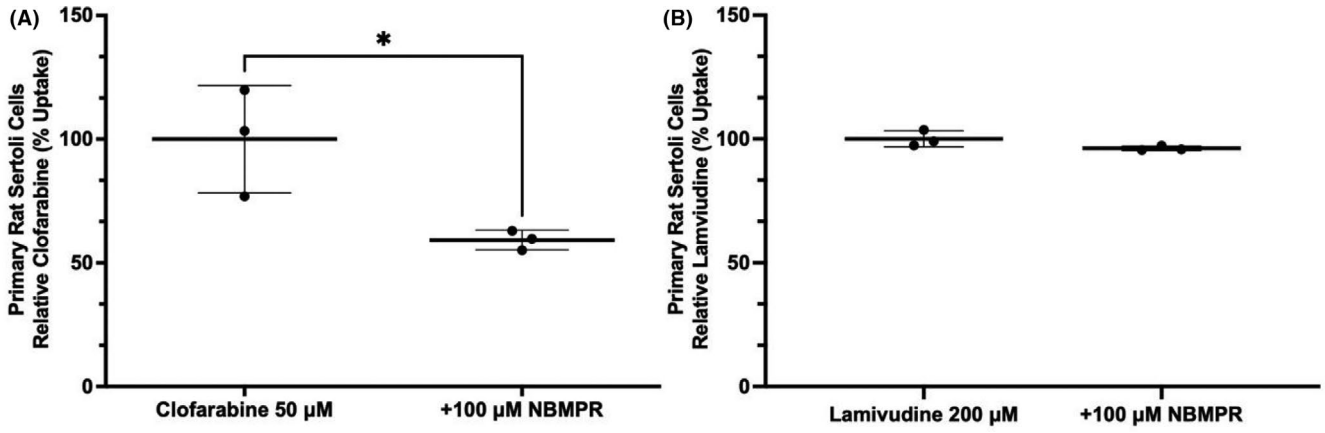

FIGURE 2 Clofarabine (A) and lamivudine (B) uptake in primary rat Sertoli cells. Cells were incubated with 50-µM clofarabine for 5 min in the presence and absence of 100-µM NBMPR, or cells were incubated with 200-µM lamivudine for 5 min in the presence and absence of 100-µM NBMPR. Data are presented as mean ± SD. Each experiment was completed in duplicate wells with $n = 3$. Each n represents separate experiments performed with Sertoli cells from biologically different rats. An unpaired, one-tailed t-test ($p \leq .05$) was used to determine differences between groups (A). An unpaired, two-tailed t-test ($p \leq .05$) was used to determine differences between groups in (B). NBMPR, 6-nitrobenzylthioinosine

TABLE 1 Plasma concentrations of clofarabine, lamivudine, and NBMPR

Plasma concentrations	Group	
Compound	**Vehicle**	**+NBMPR-P**
Clofarabine (µM)	11.12 ± 2.26	11.43 ± 1.04
NBMPR (µM)	–	9.58 ± 3.06
Lamivudine (µM)	25.82 ± 4.04	35.41 ± 6.74
NBMPR (µM)	–	7.59 ± 4.08

Note: Rats were given vehicle or 10 mg/kg NBMPR-P (IP) and then given either 10 mg/kg clofarabine or lamivudine (IV) 20 min later. Terminal plasma concentrations were determined 10 min after receiving clofarabine or lamivudine. The clofarabine + vehicle group had an $n = 6$, the clofarabine + NBMPR-P group had an $n = 5$, the lamivudine + vehicle group had an $n = 6$, and the lamivudine + NBMPR-P group had an $n = 5$. Mean plasma concentration (µM) and SD are reported.

Abbreviations: NBMPR, 6-nitrobenzylthioinosine; NBMPR-P, 6-nitrobenzylthioinosine 5′-monophosphate.

Pharmacological inhibition of the ENTs trended toward a decrease in clofarabine uptake in a human Sertoli cell line by 53% (Figure 1A) and a decrease in primary rat Sertoli cells by 40% (Figure 2A). A decrease of clofarabine uptake in the presence of 100-µM NBMPR in both isolated primary rat Sertoli cells and the human Sertoli cell line suggests that the ENTs are responsible for 40–53% of clofarabine uptake into rat and human Sertoli cells. Pharmacological inhibition of the ENTs also showed a trend toward decreased testis concentrations of clofarabine (Figure 3A). The terminal plasma concentration of NBMPR exceeded the concentration required to inhibit ENT1, the first step in nucleoside analog disposition to the male genital tract, and capable of partially inhibiting ENT2 activity.[31,58,60,61]

Interestingly, there was an increase in lamivudine plasma concentrations in the presence of NBMPR. Another study reported an increase in plasma concentrations of cytarabine in the presence of NBMPR in mice but no increase in cytarabine plasma concentrations in ENT1 −/− mice.[62] In this study and the previously mentioned study with cytarabine, NBMPR may alter the pharmacokinetic properties of tested drugs through an unknown mechanism.[62] Anderson et al. speculated there could be another unknown NBMPR sensitive transporter that altered the volume of distribution of cytarabine.[62] However, the focus of this study was to investigate the contributions of the ENTs on clofarabine disposition at the BTB and compare this to the disposition of a non-ENT substrate.

It would be ideal to study drug transepithelial transport across a polarized monolayer of primary rat or human Sertoli cells to model transport across the BTB. However, the transepithelial electrical resistance (TEER) of both rat and human Sertoli cells is low when grown on Transwell inserts, and this in vitro method for studying the BTB is not representative of the in vivo BTB.[33,52] The average TEER of human Sertoli cells is approximately 10 ohms cm^2 and approximately 40 ohms cm^2 in rat Sertoli cells, whereas other common epithelial cell lines such as Madin-Darby canine kidney or Caco-2 cells can exceed 1,000 ohms cm^2.[33,52,63] The use of Transwell inserts is not a feasible method to measure transepithelial transport of nucleosides across Sertoli cell monolayers because the low TEER demonstrates the lack of cell polarization and barrier function. Therefore, Transwell inserts were not used for this study. Previous reports in HeLa cells expressing either ENT1 or ENT2 indicated clofarabine uptake decreases in the presence of NBMPR, indicating it is a substrate of both transporters.[31] We used these methods to demonstrate that both ENTs contribute to overall uptake in rat and human Sertoli cells.

The data presented in this study and others identify clofarabine as a substrate for rat and human ENT1 and ENT2.[30,31] The rat and human orthologs of ENT1 and ENT2 are highly homologous. Rat and human ENT1 have 78.1% amino acid sequence identity, whereas rat and human ENT2 have 87.3% amino acid sequence identity.[64] The rat and

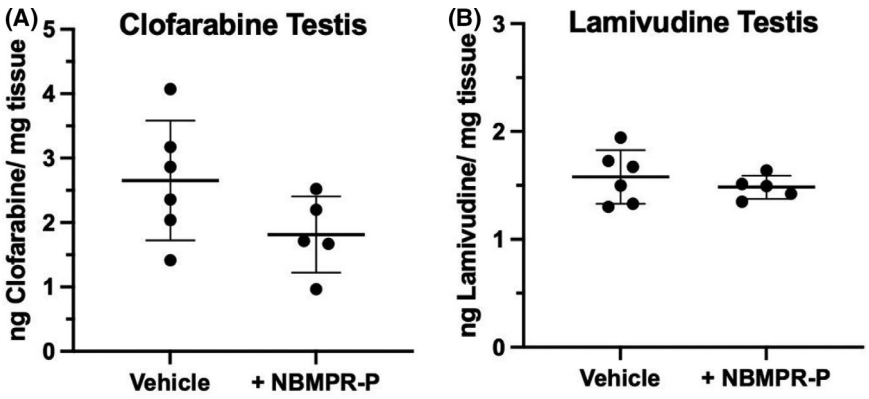

FIGURE 3 Testis clofarabine (A) and lamivudine (B) concentrations in vehicle ($n = 6$) and treatment ($n = 5$) groups. Each experiment was completed in duplicate wells with $n = 3$. Each n represents separate experiments performed from three separate passages. Data are presented as mean ± SD. An unpaired, two-tailed t-test ($p \leq .05$) was used to determine statistical differences between groups. NBMPR-P, 6-nitrobenzylthioinosine 5′-monophosphate

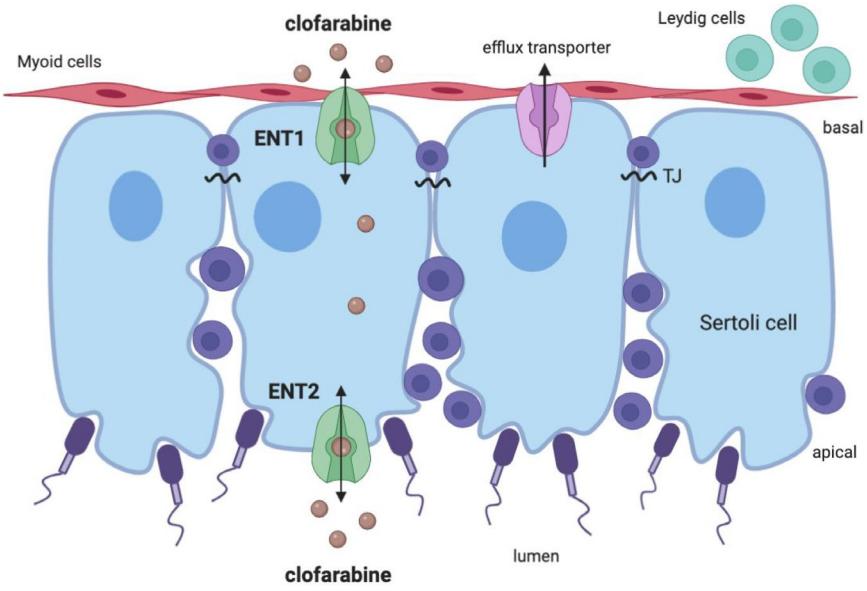

FIGURE 4 Proposed mechanism of clofarabine transport across the blood–testis barrier (BTB). Clofarabine (brown) enters Sertoli cells through ENT1 on the basal membrane. It exits Sertoli cells and enters the lumen through ENT2 on the apical membrane. Other components of the BTB such as efflux transporters (pink), tight junctions (TJ), developing germ cells, myoid cells, and Leydig cells are shown. The figure was created using BioRender.com. ENT, equilibrative nucleoside transporter

human orthologs for each transporter have similar affinities to their endogenous substrates.[44,65–69] ENT1 and ENT2 transport many of the same endogenous substrates and xenobiotics, some with differences in affinity such as with inosine and cytidine.[31,42–44,67] One limitation in using this approach includes the inability to distinguish the nonspecific binding of drug to tissue and drug penetrance into tissue, as well as the apparent alteration in lamivudine volume of distribution observed by the increase in lamivudine plasma concentration in the presence of NBMPR. However, this methodology can be used to study the disposition of other ENT substrates to the testis. Similar studies have the potential to identify mechanisms of testis penetration of other nucleoside analogs including chemotherapeutics and antivirals.

There is a possibility for unwanted drug–drug interactions with the ENTs which could impact the disposition of drugs that need to access the male genital tract (MGT). If ENT inhibitors are taken with ENT substrates, there is a potential for an increase in exposure to the substrate or failure to reach adequate therapeutic concentrations within the MGT. Currently used chemotherapeutics are substrates of the ENTs, including gemcitabine, cytarabine, and fludarabine.[30,48,70–72] This also applies to treatment of viruses including HIV because nevirapine, darunavir, and abacavir are known ENT inhibitors.[31,42,72] There is limited information on currently prescribed drugs that are known ENT substrates and inhibitors; therefore, it is necessary to continue to identify ENT substrates and inhibitors to reveal the full potential of drug–drug interactions.[72]

Biological barriers and drug resistance may contribute to poor treatment response or cancer relapse. These limitations lead to more aggressive treatment options. Typical treatment options involve radiation therapy or orchiectomy [7,8] which leave the patient with reduced or complete loss of fertility. These treatment options are employed to great success, although at significant cost to the reproductive capacity of these patients. Therefore, it is desirable to avoid radiation therapy or orchiectomy to prevent the loss of fertility and the hormone replacement therapy that begins following treatment. Chemotherapeutics such as clofarabine are a less drastic solution to treating these cancers, and their toxicological effects on fertility

may be reversible compared with radiation therapy or orchiectomy. Currently, clofarabine is recommended as a third-line therapy for ALL after two other types of treatments have failed. In this study, clofarabine is shown to enter Sertoli cells and accumulate in the testes, where cancer cells may also reside and cause future relapse due to the presence of the BTB. Because clofarabine is capable of reaching this sanctuary site, it may be effective as a first-line treatment in order to avoid testicular relapse of ALL in children and adolescents and adult AML patients that want to remain fertile.

DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Participated in research design: SRM, JLJ, EQJ, JJG, SHW, NJC. Conducted experiments: SRM, JLJ, MEM, RKH, EQJ. Performed data analysis: SRM, JLJ, EQJ, JJG, NJC. Wrote or contributed to the writing of the manuscript: SRM, JLJ, MEM, RKH, EQJ, JJG, SHW, NJC.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings within this study are available within this manuscript.

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