Interaction of the Novel Adenosine Uptake Inhibitor 3-[1-(6,7-Diethoxy-2-morpholinoquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1H,3H)-quinazolinenedione Hydrochloride (KF24345) with the es and ei Subtypes of Equilibrative Nucleoside Transporters

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ABSTRACT

Nucleosides such as adenosine, as well as many nucleoside-based drugs, permeate cell membranes via a family of equilibrative nucleoside transporters (ENTs). We assessed the effects of 3-[1-(6,7-diethoxy-2-morpholinoquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1H,3H)-quinazolinenedione hydrochloride (KF24345), a novel anti-inflammatory agent that potentiates the actions of adenosine, on the es (inhibitor-sensitive) and ei (inhibitor-resistant) subtypes of ENTs in human, mouse, and rat cells. KF24345 was similar to the prototypical high-affinity inhibitor nitrobenzylthioinosine (NBMPR) for blocking the human es transporter (K_i of ~0.4 nM), but was 50-fold more effective than NBMPR at blocking the human ei transporter (K_i of ~100 nM). KF24345 displayed significantly less species heterogeneity in its affinity for the es transporter than did dipyridamole, a widely used inhibitor of nucleoside transport; KF24345 may thus prove useful as an inhibitor for studies of nucleoside metabolism in a range of animal models. Furthermore, KF24345 seemed to act as a noncompetitive inhibitor of both [3H]NBMPR binding and [3H]nucleoside uptake by human es transporters, and these kinetics were consistent with an observed slow dissociation of KF24345 from the inhibitor binding site. KF24345 also exhibited unusual biphasic profiles for inhibition of [3H]NBMPR binding to membranes prepared from a recombinant human es transporter model (PK15-hENT1), suggesting the presence of multiple populations of NBMPR binding proteins in these membranes. The atypical tight binding interaction of KF24345 with the es transporter may prove useful for the molecular delineation of inhibitor binding domains and will facilitate its use as an in vivo inhibitor of nucleoside transport in studies focused on the biological effects of adenosine.

Nucleosides permeate cell membranes via two distinct families of transport proteins, classified as concentrative (Na^+-dependent) or equilibrative (Na^+-independent) (Baldwin et al., 1999; Hyde et al., 2001; Ritzel et al., 2001). The equilibrative nucleoside transporters (ENTs), which are the most broadly expressed family, can be functionally divided into two subtypes based on their sensitivities to inhibition by nitrobenzylthioinosine (es, NBMPR-sensitive; ei, NBMPR-insensitive) (Thorn and Jarvis, 1996). Many mammalian cells concurrently express both the es and ei subtypes of ENTs, and representative gene products (designated ENT1 and ENT2, respectively) have been cloned from human (Griffiths et al., 1997), rat (Yao et al., 1997), mouse (Kiss et al., 2000), and dog (J. R. Hammond, M. Stolk, R. G. E. Archer, and K. McConnell, submitted for publication) tissues. The ei system is weakly inhibited by compounds such as NBMPR, dilazep, and drafazine at concentrations in the high nanomolar or micromolar range. In contrast, the es subtype is blocked almost completely by these agents at concentrations of less than 10 nM (Thorn and Jarvis, 1996; Hyde et al., 2001). The high affinity and selectivity of NBMPR for the es transporter has enabled extensive use of [3H]NBMPR as a specific probe for this system (Jarvis and Young, 1980; Jarvis et al., 1982b; Young and Jarvis, 1985; Hammond, 1991). Indeed, the K_i for inhibition of the binding of [3H]NBMPR by a compound often reflects its capacity to inhibit substrate flux via the es transporter (Jarvis et al., 1982a; Hammond, 2000).

ABBREVIATIONS: ENT, equilibrative nucleoside transporter; es, equilibrative inhibitor-sensitive; NBMPR, nitrobenzylmercaptopurine riboside (nitrobenzylthioinosine); ei, equilibrative inhibitor-insensitive; NBTGR, nitrobenzylthioguanosine; FBS, fetal bovine serum; ECGS, endothelial cell growth supplement; DMEM, Dulbecco’s modified Eagle’s medium; MVVEC, rat microvascular endothelial cell; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; MEM, minimum essential medium.
The principal endogenous substrate for ENTs is adenosine. Adenosine has been implicated in the control of cardiovascular and neuronal excitability and is well established as an effective vasodilator in both the central nervous system and peripheral vasculature (Dunwiddie and Masino, 2001; Mubagwa and Flameng, 2001; Tabrizchi and Bedi, 2001). Adenosine has also been reported to have significant anti-inflammatory activity in various models (Cronstein, 1994; Linden, 2001). These actions of adenosine are produced through a family of G protein-coupled receptors in the plasma membrane of cells. Adenosine is metabolized predominantly by intracellular enzymes subsequent to its cellular accumulation by ENTs, thereby terminating its actions at these extracellular receptor sites (Arch and Newsholme, 1978). Hence, compounds that block adenosine uptake potentiate the receptor-mediated physiological effects of adenosine (Van Belle, 1993; Griffith and Jarvis, 1996).

Noji and coworkers (Noji et al., 2002a,b,c) have recently reported on the anti-inflammatory activity of a new quinazolinedione derivative, (3-[1-(6,7-diethoxy-2-morpholinoquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1H,3H)quinazoloninedione hydrochloride, designated KF24345 (Fig. 1). These investigators proposed that the anti-inflammatory activity of this compound was due to inhibition of adenosine uptake and metabolism, and that it was more effective than existing agents such as dilazep, dipyridamole, and drafalazine due to enhanced bioavailability after oral administration and a longer duration of action in vivo (Noji et al., 2002c). However, these studies did not distinguish between ENT subtypes. Therefore, the present study was undertaken to compare the effectiveness of KF24345 as an inhibitor of both the es and ei transporters in a number of cell types. Cells derived from human, mouse, and rat were used in recognition of the well established species heterogeneity in the sensitivity of the es transporter to a number of inhibitors (Hammond and Clanagan, 1985; Oggunade and Baer, 1990). We also determined the type of inhibition produced by KF24345 and assessed its capacity to modify the dissociation of [3H]NBMPR from its high-affinity sites on the es transporter via interactions with previously reported (Jarvis et al., 1983; Hammond, 1991) allosteric binding sites on the transporter.

Materials and Methods

**Chemicals.** [G-3H]Formycin B (14 Ci/mmol) and [G-2H]NBMPR (35–50 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA), and [3H]water (1 mCi/g) was purchased from DuPont Canada Inc. (Markham, ON, Canada). KF24345 was generously provided by Dr. Noji (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan). Nonradiolabeled formycin B, NBMPR, nitrobenzylthioguanosine (NBGTGR), dipyridamole [2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-d]pyrimidine], collagenase, trypsin, bovine serum albumin, and protease inhibitor cocktail [4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, E-64] were supplied by Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Media 199 with Earle’s salts and L-glutamine (M199), MEM, Dulbecco’s modified Eagle’s medium (DMEM), trypsin-EDTA, penicillin, streptomycin, amphotericin B, and heparin were from Invitrogen (Burlington, ON, Canada). Lectin GS-1-B4 of *Griffonia simplicifolia* was purchased from Calbiochem (La Jolla, CA), and dispase was obtained from Roche Applied Science (Indianapolis, IN). Magnetic beads (Dynabeads M-450 Epoxy) and magnet (MPC1) were purchased from Dynal Biotech (Lake Success, NY). Endothelial cell growth supplement (ECGS) was supplied by BD Biosciences (Oakville, ON, Canada). All compounds used were of reagent grade.

**Cell Culture.** Mouse C2C12 myoblasts (obtained from Dr. I. Skerjanec, University of Western Ontario, London, ON, Canada) and human osteosarcoma (U2-OS) cells (obtained from Dr. D. Litchfield, University of Western Ontario) were cultured in DMEM, containing 10% FBS plus antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin), at 37°C in a 5% CO₂ humidified environment. PK15-NDT (nucleoside transport deficient) cells stably transfected with human ENT1 or ENT2 transporters (Ward et al., 2000) were generously provided by Dr. Ming Tse (Johns Hopkins University, Baltimore, MD) and cultured in MEM containing 5% FBS and antibiotics at 37°C, 5% CO₂.

Rat microvascular endothelial cells (rMVECs) were isolated from the extensor digitorum longus muscles of male Wistar rats (250–300 g) using a protocol adapted from Wilson et al. (1996). Tissues were removed under anesthesia by sodium pentobarbital (35 mg/ml, 0.6 ml/500 g body weight), and finely minced in Krebs-Ringer solution (127 mM NaCl, 4.6 mM KCl, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, 8.3 mM d-glucose, 24.8 mM NaHCO₃, 2 mM pyruvate, 11.4 mM creatinine, 20 mM taurine, 5 mM d-ribose, 2 mM L-asparagine, 2 mM L-glutamine, 1 mM L-arginine, 0.5 mM uric acid). The tissue suspension was placed in a water-jacketed organ bath (50 ml, 37°C) containing collagenase (0.84 mg/ml), trypsin (0.12 mg/ml), dispase (0.12 mg/ml), and bovine serum albumin (1.6 mg/ml), and bubbled with 5% CO₂ for 45 min at a flow rate that circulated tissue gently in the organ bath. The enzyme-treated tissue was filtered through 100-µm

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**Fig. 1.** Chemical structures of KF24345, dipyridamole, and NBMPR.
nylon mesh into sterile 50-ml tubes containing M199 (37°C). Dissociated cells in the filtrate were pelleted (5 min at 150g), washed twice with M199, resuspended in a 14 ml of M199 containing 2 × 10^7 GS-1 leguminous bacteria/ml, and incubated on a rotating spindle for 10–15 min at 37°C. The tube was then placed in a magnet for 2 min, and M199 was removed by using a low-pressure vacuum aspirator. Beads magnetically bound to the wall of the tube were washed twice with M199, resuspended in 6 ml of M199 containing 20% FBS, and transferred to a six-well plate for culture. Endothelial cell isolation was confirmed (>95% purity) by immunohistochemical staining for von Willebrand Factor VIII as described by Wilson et al. (1996). After three passages, rMVECs were cultured in T-175 flasks in M199 plus 10% FBS supplemented with antibiotic/antimycotic (100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B), L-glutamine (0.30 mg/ml), endothelial cell growth supplement (30 μg/ml), and heparin (2.5 units/500 ml) and maintained in a humidified atmosphere of 5% CO_2 at 37°C.

For experimental procedures, cells were removed from flasks by trypsinization [0.5% (w/v), 10 min, 37°C] and then diluted at least 4-fold with culture media + 10% FBS and pelleted by centrifugation. Cell pellets were washed once by resuspension/centrifugation in Dulbecco’s phosphate-buffered saline (PBS; 137 mM NaCl, 6.3 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM MgCl_2·6H_2O, 0.9 mM CaCl_2·H_2O; pH 7.4) and then diluted to the appropriate cell concentration for the assays described below.

Membrane Preparations. Crude membranes were prepared from U2-OS and PK15 cells as described by Tse and coworkers (Ward et al., 2000). Cells were lysed by sonication in 5 mM Na_2HPO_4 (pH 8) containing a cocktail of protease inhibitors. The cell lysate was then centrifuged for 10 min at 3000g followed by centrifugation of the resulting supernatant for 30,000g. The final pellet was fine-needle homogenized in 5 mM Na_2HPO_4 buffer and stored at −80°C (~1 mg protein/ml).

Human erythrocyte ghost membranes were prepared as described by Dodge et al. (1963). Whole blood was centrifuged at 1000g for 10 min and the plasma and buffy coat removed. Erythrocytes were then washed four times (40,000 g/ml) and resuspended in a 14 ml of M199 containing 10% FBS and pelleted by centrifugation. The ability of a range of concentrations of dipyridamole and KF24345 to inhibit the influx of [3H]formycin B via the es and ei transporters was assessed by incubating cells with the test inhibitors, ≥100 nM NBMPR to selectively block es transporters (Hammond, 2000). Non-mediated [3H]formycin B uptake, which was quantified as total minus nonspecific binding, was subtracted from all data before analysis, was measured as that accumulated by cells in the presence of 10 μM NBTRG and 10 μM dipyridamole. Inhibition constants (IC_{50}) were calculated from best-fit sigmoid curves (GraphPad Prism version 4; GraphPad Software Inc., San Diego, CA) representing the relationship between the transporter-mediated uptake of [3H]formycin B and the log of the inhibitor concentration. Identical procedures were used in those experiments where [3H]uridine was used as the substrate instead of [3H]formycin B.

[3H]NBMPR Binding. Binding assays were performed at room temperature in PBS. In some cases (see Results), 0.01% 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate [CHAPS, (w/v)] was added to the buffer to prevent nonspecific association of inhibitor with the glass incubation tubes and filters (Hammond, 2000). For equilibrium binding assays, incubations (final volume 1 ml) were initiated by adding cell suspension (10^5–10^6 cells) or isolated membranes (5–50 μg of protein), [3H]NBMPR (≥100 nM), and protein concentrations were chosen such that ligand depletion was minimized (<12% in all cases), while maintaining a specific-to-nonspecific binding ratio of two or greater for all [3H]NBMPR concentrations used. Incubations were terminated after a defined time (45 min for mass law analyses; 60–90 min for inhibition studies) by dilution with 5 ml of ice-cold 10 mM Tris-HCl (pH 7.1) followed by rapid filtration through Whatman GF/B filters using a modified Brandel cell harvester. Filters were then washed once with 5 ml of ice-cold 10 mM Tris-HCl and placed in 5 ml of BCS liquid scintillation fluid (Amersham Biosciences, Oakville, ON, Canada) for analysis of radioactive content. Nonspecific binding of [3H]NBMPR was determined as that remaining membrane-associated in the presence of 10 μM NBTRG. Specific binding was defined as total minus nonspecific binding. In all cases, K_d and B_{max} values were derived from hyperbolic curves fitted to plots of specific binding versus equilibrium-free concentration of [3H]NBMPR. Inhibition constants (IC_{50}) for dipyridamole and KF24345 were calculated from variable slope sigmoid curves fitted to the log of the inhibition constant. In some cases, data were fitted to both one-site and two-site models and the best fit determined based on the F test (P < 0.05; GraphPad Prism version 4). K_d values were calculated from IC_{50}, according to the equation of Cheng and Prusoff (1973) using K_d values previously defined for [3H]NBMPR binding to the cell and membrane preparations used. In some cases, K_d values were determined directly via double reciprocal plot analyses (1/bound versus 1/[NBMPR]); the slopes of the regression lines from the double reciprocal graphs were plotted (ordinate) against the inhibitor concentrations (abscissa), and K_d values were determined as the negative x-intercepts of these secondary plots.

For binding dissociation analyses, erythrocyte membranes (20 μg/ml) or PK17-eENT membranes (160 μg/ml) were incubated with [3H]NBMPR (1.5 nM) for 45 min at room temperature and then dipyridamole, NBTRG, or KF24345 (1.5 μM final concentration) was added to initiate dissociation. Aliquots (500 μl) of these mixtures were filtered, as described above, at defined times after addition of displacer. Nonspecific binding of [3H]NBMPR was defined as that which remained membrane-associated 60 min after addition of 1.5 μM NBTRG and was subtracted from all data points before further analysis.

Results

[3H]Formycin B Uptake. The relative amount of es- to ei-mediated transport activity in each cell line was based on the differential sensitivities of [3H]formycin B uptake to in-
hibition by NBMPR. Human U2-OS osteosarcoma cells and rMVECs accumulated $10 \mu M$ $[^3H]$formycin B via both the $es$ and $ei$ systems with similar alacrity (53 ± 3 and 53 ± 4% $es$, respectively) (Fig. 2, A and C), whereas the mouse C2C12 myoblasts expressed predominantly the $es$ type of transporter (16 ± 4% $ei$-mediated uptake) (Fig. 2D). We also confirmed, based on NBMPR sensitivity, that the PK15-hENT1 and PK15-hENT2 stable transfectants expressed the expected transporter subtype (data not shown).

**U2-OS Cells.** KF24345 inhibited the uptake of $[^3H]$formycin B by U2-OS cells in a biphasic manner (Fig. 2A) with $IC_{50}$ values of 0.38 and 107 nM for the high- and low-affinity components, respectively, in a ratio (42 ± 5% high affinity) similar to that seen for NBMPR (Table 1). When cells were preincubated with 100 nM NBMPR to selectively block the $es$-mediated uptake of $[^3H]$formycin B, the remaining $ei$-mediated uptake was inhibited by KF24345 with an $IC_{50}$ of 92 nM; this value was not significantly different from the lower affinity KF24345 inhibition component observed in the cells not treated with NBMPR ($IC_{50} = 107$ nM; Fig. 2A; Table 1).

**PK15-hENT1/hENT2.** KF24345 inhibited the uptake of $[^3H]$formycin B by the PK15-hENT1 stable transfectants in a manner consistent with the presence of only a single transporter subtype (Fig. 2B). However, the $IC_{50}$ value (12 nM) for KF24345 inhibition of formycin B uptake in the PK15-hENT1 cells was 30-fold higher than that obtained for inhibition of the $es$ transporter in U2-OS cells (0.38 nM). Likewise, formycin B uptake by the PK15-hENT2 ($ei$-phenotype) transfectants was inhibited by KF24345 with an $IC_{50}$ of 1100 nM, which is, again, 10-fold higher than that obtained for KF24345 inhibition of the $ei$ transporter in the U2-OS cells (Table 1). Dipyridamole was similar to KF24345 with an $IC_{50}$ value of 10 ± 5 nM for inhibiting formycin B uptake by PK15-hENT1 (Fig. 2B).

**C2C12 Cells.** KF24345 inhibited $[^3H]$formycin B uptake by mouse C2C12 cells in an essentially monophasic manner with an $IC_{50}$ value of 32 nM (Table 1). This contrasts with NBMPR, which inhibited uptake in the C2C12 cells in a biphasic manner with 16% of the transporter-mediated uptake being only weakly sensitive to NBMPR (Fig. 2D).

**MVECs.** Rat MVECs were similar to the U2-OS cells in that they showed a biphasic profile for inhibition of the total mediated uptake of $[^3H]$formycin B by KF24345 (Fig. 2C) with 35 ± 5% of the uptake inhibited with an $IC_{50}$ value of 0.15 nM, and the remaining 65% inhibited by KF24345 with an $IC_{50}$ value of 700 nM (Table 1). This ratio of high-to-low-affinity KF24345-sensitive components was significantly different from the ratio obtained using NBMPR as the inhib-

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**Fig. 2.** Inhibition of $[^3H]$formycin B uptake by U2-OS, PK15-hENT1/hENT2, mVEC, and C2C12 cells. A range of concentrations of KF24345 (■) were tested for their capacities to inhibit the total ($es + ei$) and NBMPR-resistant ($ei$, $ei$) transporter-mediated influx of $10 \mu M$ $[^3H]$formycin B (15 s). B, data for KF24345 inhibition of $10 \mu M$ $[^3H]$formycin B uptake (15 s) by PK15 cells stably transfected with either hENT1 or hENT2. The established transport blockers dipyridamole (▼) and/or NBMPR (○) were tested in parallel for their capacities to inhibit the total transporter-mediated uptake of $[^3H]$formycin B by the indicated cell types. Results are shown as a percentage of the accumulation observed in the absence of test inhibitor (control) after subtraction of the nonmediated uptake component. The average total/nonmediated uptake (picomoles per assay) ratios were 5.7:1.1, 4.2:2.3, 13.1:3.7, 6.7:2.7, and 3.9:1.6 for the U2-OS, PK15-hENT1, PK15-hENT2, C2C12, and mVECs, respectively. Each point represents the mean ± S.E.M. from at least five experiments conducted in duplicate. $IC_{50}$ values derived from these experiments are shown in Table 1.
itor (53 ± 4% high affinity; Fig. 2C). Dipyridamole inhibited formycin B uptake by rMVECs, with an IC50 of 410 ± 25 nM (Fig. 2C) and a pseudo Hill coefficient (nH) of 0.52 ± 0.02.

[3H]NBMPR Binding. To further assess the interaction of KF24345 with the es nucleoside transporter, we used the high-affinity es-selective probe [3H]NBMPR. Each of the isolated membrane preparations (from human erythrocytes, PK15-hENT1, or U2-OS cells), as well as intact U2-OS cells and C2C12 cells, bound [3H]NBMPR to an apparent single class of site, as evidenced by linear Scatchard plots (Fig. 3, A and B) and Hill coefficients not different from unity (Table 2).

However, binding to rMVECs resulted in statistically significant (F test; P < 0.05) curvilinear Scatchard plots (Fig. 3C), possibly reflecting multiple binding site populations or compartmentalization of the radioligand. The high-affinity [3H]NBMPR binding component in rMVECs (KD = 0.10 nM), which represented 61% of the total binding capacity (Table 2), was similar to that seen in the other cell types.

KF24345 inhibited the binding of [3H]NBMPR to mouse C2C12 cells, rat MVECs, and membranes prepared from human erythrocytes and U2-OS cells, to yield relatively monophasic profiles (Fig. 4, A and C) from which KD values of 9.1, 7.3, 1.3, and 0.62 nM, respectively, were derived (Table 3). In contrast, PK15-hENT1 membranes exhibited a clearly biphasic inhibition profile for KF24345 inhibition of [3H]NBMPR binding (Fig. 4A). The larger component had a KD value of 0.74 nM for KF24345, whereas the smaller binding component was almost three orders of magnitude more sensitive to KF24345 (KD = 1.5 × 1000 nM; Table 3). Dipyridamole, assessed in parallel, inhibited [3H]NBMPR binding in a manner that did not readily distinguish between multiple binding components in each of the cell models tested and generally had a higher affinity for the human cell membranes relative to the mouse C2C12 or rat cells (Fig. 4, B and D; Table 3). When these studies were extended over a range of [3H]NBMPR concentrations, KF24345 was found to behave as a noncompetitive inhibitor of [3H]NBMPR binding to both human erythrocyte membranes and U2-OS membranes (Fig. 5), decreasing binding Bmax in a concentration-dependent manner (repeated measures analysis of variance; P < 0.05), with no significant effect on KD (Table 4). KD values determined directly from these experiments (0.53 ± 0.11 and 1.8 ± 0.7 nM for erythrocyte membranes and U2-OS membranes, respectively) were of a similar magnitude to those derived, using the Cheng-Prusoff equation, from the inhibition profiles shown in Fig. 4A (Table 3). Apparent noncompetitive inhibition kinetics were observed both with and without preincubation of the membranes with KF24345, as well as in the presence or absence of 0.01% CHAPS to reduce potential nonspecific interactions of the inhibitor with the assay tubes (Hammond, 2000). Likewise, preincubation of the membranes with [3H]NBMPR for 30 min before the addition of KF24345 did not change the inhibition type or the KD value significantly (data not shown).

Previous studies have shown that inhibition of [3H]NBMPR binding tends to mirror the effects of inhibitors on nucleoside uptake via the es transporter. We therefore assessed the mode of inhibition produced by KF24345 on the rate of uptake of a range of [3H]formycin B or [3H]uridine concentrations in U2-OS cells or PK15-hENT1 cells, respectively (Fig. 6). [3H]Uridine was used as the substrate in PK15-hENT1 cells based on preliminary data suggesting that formycin B is a poor substrate for the recombinant hENT1 system in these cells. In both cell lines, 10 nM KF24345 decreased the Vmax of radiolabeled substrate uptake by the es/ENT1 transporter with no significant effect on Kmax (Table 4), consistent with a noncompetitive type of inhibition of the translocation process.

The next step was to determine whether the apparent noncompetitive inhibition profile was due to slow dissociation of the KF24345 relative to [3H]NBMPR at a single site (tight binding inhibitor) or due to reversible interactions with a mutually exclusive but distinct binding site. Human erythrocyte membranes (~1 mg of membrane protein) were incubated in either the presence or absence of 100 nM KF24345 or 50 nM NBTGR (concentrations ~100× KD; inhibiting the binding of [3H]NBMPR) for 60 min and then washed in a controlled manner (two washes with 1.5 ml of buffer/ml protein, 10-min incubation) before assessment of the binding of [3H]NBMPR. Preincubation with NBTGR did not modify significantly the KD or Bmax of [3H]NBMPR binding. However, preincubation with KF24345 resulted in a 62% reduction in binding Bmax (from 0.50 ± 0.04 to 0.19 ± 0.06 pmol/mg protein) with no significant change in Kmax (1.1 ± 0.2 and 1.5 ± 0.7 nM for the control and KF24345-treated membranes, respectively) (Fig. 7).

Allosteric Effects of KF24345 on NBMPR Binding. The presence of an allosteric binding site on the es transporter through which compounds can modify the affinity of the transporter for NBMPR has been postulated by our laboratory (Hammond, 1991) and others (Jarvis et al., 1983; Shi and Young, 1986). Displacement of [3H]NBMPR from its binding sites in human erythrocyte membranes with NBTGR (1.4 μM) resulted in a dissociation rate (k−1) of 0.357 ± 0.017 min−1 (Fig. 8). Dipyridamole decreased the rate of dissociation-
KF24345 and dipyridamole had similar effects on the dissociation of [3H]NBMPR from PK15-hENT1 membranes (Fig. 8B). However, unlike that seen for the erythrocyte membranes where the dissociation was monophasic, [3H]NBMPR dissociated from the PK15-hENT1 membranes in a multiphasic manner. When using KF24345 as the displacer, 40 ± 12% of the bound [3H]NBMPR dissociated rapidly at a rate of 0.30 ± 0.02 min⁻¹, whereas the remaining radioligand dissociated more slowly at a rate of 0.14 ± 0.03 min⁻¹. NBTGR resulted in a similar proportion of rapid to slow-dissociating components (45 ± 10% rapid dissociation) with a faster rate for both components relative to that seen using KF24345 (0.40 ± 0.02 and 0.26 ± 0.03 min⁻¹). In contrast, using dipyridamole as the displacer, only 24 ± 7% of the binding was relatively rapidly dissociating (0.13 ± 0.02 min⁻¹), with the remaining ligand dissociating at a very low rate of 0.031 ± 0.016 min⁻¹. It is noteworthy that the slower dissociating components in PK15-hENT1 membranes had rate constants in the presence of dipyridamole and KF24345 that were identical to those derived using these agents as displacers in the human erythrocyte membranes.

**Discussion**

Human U2-OS cells expressed both ei and es transporters, and KF24345 inhibited [3H]formycin B uptake by these cells in a biphasic manner compatible with a differential affinity for es versus ei. Our data showed that the high-affinity component of KF24345 inhibition of [3H]formycin B uptake likely represents interactions with the es transporter. These data also indicated that while KF24345 had a significantly (300-fold) lower affinity for the ei transporter of U2-OS cells relative to es, it was still significantly more effective than NBMPR in this regard. The selectivity of KF24345 for the human es transporter was confirmed using PK15 cells transfected with either hENT1 (es) or hENT2 (ei). However, the IC₅₀ values obtained for inhibition in the PK15-hENT transfecants were about 10-fold higher than those observed for KF24345 inhibition of es- and ei-mediated uptake by the endogenous transporters in human U2-OS cells. This difference may reflect atypical post-translational processing of the recombinant hENT1 protein by the PK15 epithelial cells. For example, it has been reported that the glycosylation state of the transporter affects inhibitor affinity (Vickers et al., 1999).

Rat and mouse es transporters typically have a lower affinity for many inhibitors compared with the human transporter (Hammond and Clanachan, 1985; Ogbunude and Baer, 1990). This was seen for dipyridamole in the present study (compare rat MVECs with PK15-hENT1 cells in Figs. 2 and 4). Although the affinity of the mouse es transporter for KF24345 was indeed less than that seen for the human cell models, the difference was not as great as that seen for dipyridamole. Furthermore, the high-affinity component of KF24345 inhibition of formycin B uptake in rat MVECs was similar to that seen for human cells. This makes KF24345 one of the more potent inhibitors currently available for the rat es transporter. Interestingly, the ratios of high-/low-affinity KF24345 inhibition components in both the rat MVECs (35% high affinity) and mouse C2C12 cells (100% high affinity) were different from the es/ei ratios derived using NBMPR (53 and 84% es, respectively). The reasons for these differences are obscure, but a similar result has been reported for
TABLE 2

Mass law analyses of [3H]NBMPR binding to various cell types

<table>
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<tr>
<th>Membranes</th>
<th>K_D (nM)</th>
<th>B_max (pmol/mg)</th>
<th>n_H</th>
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<tr>
<td>Erythrocyte</td>
<td>0.73 ± 0.02</td>
<td>28 ± 2</td>
<td>1.02 ± 0.01</td>
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<tr>
<td>PK15-hENT1</td>
<td>0.17 ± 0.02</td>
<td>1.9 ± 0.3</td>
<td>1.07 ± 0.03</td>
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<tr>
<td>U2-OS</td>
<td>0.29 ± 0.03</td>
<td>2.5 ± 0.1</td>
<td>1.00 ± 0.01</td>
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</table>

TABLE 3

KF24345 and dipyridamole inhibition of [3H]NBMPR binding to membranes prepared from human erythrocytes, human osteosarcoma (U2-OS) cells, and PK15 cells stably transfected with hENT1, as well as intact mouse C2C12 myoblasts and rat MVECs

<table>
<thead>
<tr>
<th>Component</th>
<th>K_D (nM)</th>
<th>B_max (pmol/mg)</th>
<th>n_H</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS</td>
<td>1.3 ± 0.2</td>
<td>1.30 ± 0.13</td>
<td></td>
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<tr>
<td>hRBC</td>
<td>0.62 ± 0.17</td>
<td>0.95 ± 0.03</td>
<td></td>
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<tr>
<td>PK15-hENT1 (A)</td>
<td>0.74 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK15-hENT1 (B)</td>
<td>0.0015 ± 0.0007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2C12</td>
<td>9.1 ± 0.6</td>
<td>0.92 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>MVEC</td>
<td>7.3 ± 0.5</td>
<td>0.79 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Inhibition of [3H]NBMPR binding to U2-OS, PK15-hENT1, and human erythrocyte membranes (A and B), and C2C12 cells and rMVECs (C and D), by KF24345 and dipyridamole. Cells or membranes were incubated with a range of concentrations of KF24345 (A and C) or dipyridamole (B and D) and [3H]NBMPR (0.6 nM) for 60 min and then processed as described in the text. Data are shown as percentage of control binding where the “control” was the site-specific binding of [3H]NBMPR in the absence of inhibitor. The average total/nonspecific binding (femtomoles per assay) ratios were 35.2, 29.6, 32.9, 57.5, and 58.7 for the erythrocyte, U2-OS, PK15-hENT1, C2C12, and rMVEC preparations, respectively. Each point represents the mean ± S.E.M. from at least five experiments conducted in duplicate.

dilazep inhibition of uridine uptake by Ehrlich ascites cell es and ei transporters (Hammond, 1994). This may reflect inhibitor-induced modifications of substrate affinity for the two transporter subtypes, or a change in the relative functional stability of the transporters in the cell membrane. In general, though, KF24345 interacts with the es transporter in a manner more similar to NBMPR than dipyridamole, but it is more like dipyridamole in its interactions with the ei transporter.

To further assess the interaction of KF24345 with the es
transporter, we analyzed the ability of this novel compound to interfere with the binding of [3H]NBMPR to its high-affinity sites on the transporter (Thorn and Jarvis, 1996; Hyde et al., 2001). KF24345 was a potent inhibitor of the binding of [3H]NBMPR to all of the cell lines tested. The human cells had the highest affinity, followed by mouse C2C12 cells and rat MVECs. In C2C12 cells and U2-OS membranes, the $K_i$ for KF24345 inhibition of NBMPR binding reflected its potency for inhibiting es-mediated formycin B uptake. However, in PK15-hENT1 cells, NBMPR binding was 16 times more sensitive to KF24345 than was [3H]formycin B uptake. The opposite was true for rMVECs, where the NBMPR binding site was almost 50 times less sensitive to KF24345 than was the high-affinity component of [3H]formycin B uptake (Tables 1 and 3). Both of the aforementioned cell lines had other unusual characteristics; NBMPR binding to PK15-hENT1 membranes was inhibited in a biphasic manner by KF24345, and the rMVECs appeared to have two distinct NBMPR binding components (Fig. 3C). The relatively low pseudo Hill coefficient obtained for KF24345 inhibition of NBMPR binding in the rMVECs ($n_H = 0.79$) also implies that one KF24345 binding component in these cells. rMVECs may express a proportion of their NBMPR binding proteins in intracellular membranes, similar to that reported for BeWo cells (Mani et al., 1998). These intracellular sites may have different inhibitor sensitivities relative to the transporter in the plasma membrane.

This line of reasoning does not, however, explain the biphasic profile for KF24345 inhibition of [3H]NBMPR binding to membranes prepared from PK15-hENT1 cells; these membranes had only a single binding site for [3H]NBMPR (Fig. 3A). This biphasic profile was unique to KF24345 because dipyridamole, assessed in parallel, inhibited binding in a monophasic manner. The PK15-hENT1 cells do not express any other es-like transporter besides the recombinant hENT1 (Ward et al., 2000), and the parent nucleoside transporter-deficient cell line has no binding sites for [3H]NBMPR (Ward et al., 2000; J. R. Hammond and M. Stolk, unpublished data). Although it remains possible that the incubation time used for these studies was not sufficient to allow complete equilibration of all ligands with the es transporter, this seems unlikely because preincubation of membranes for 30 min with KF24345 before exposure to [3H]NBMPR for an additional 60 min gave results that were indistinguishable from those obtained by simultaneous exposure of the membranes to KF24345 and [3H]NBMPR for 60 min. Therefore, [3H]NBMPR may indeed be binding to a heterogenous population of sites in the PK15-hENT1 cell membranes with differential affinities for KF24345, but similar affinities for other inhibitors such as NBMPR and dipyridamole. The higher affinity component ($K_i = 1.5$ pM) was the atypical one because the lower affinity site had a $K_i$ for KF24345 (0.74 nM) similar to

**TABLE 4**

Effect of KF24345 on the kinetics of [3H]nucleoside uptake and [3H]NBMPR binding by the es/ENT1 transporter of human erythrocytes, U2-OS cells, and PK15-hENT1 cells

[3H]NBMPR binding and [3H]nucleoside flux were measured in the absence (control) and presence of the indicated concentrations of KF24345 (KF) as described in Figs. 5 and 6, respectively. The values shown were derived from best-fit hyperbolic curves to nontransformed specific binding data; $B_{max}$ and $V_{max}$ represent the maximum number of binding sites and the maximum substrate uptake velocity, respectively. $K_d$ and $K_s$ represent the apparent binding affinity of [3H]NBMPR and the apparent affinity of the [3H]nucleoside substrate, respectively, for the es transporter. Each value is the mean ± S.E.M. from the number of experiments shown in parentheses.

<table>
<thead>
<tr>
<th>Binding</th>
<th>U2-OS Membranes (n = 5)</th>
<th>Erythrocyte Membranes (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_{max}$</td>
<td>Control 0.6 nM KF 2.5 nM KF</td>
<td>Control 1.2 nM KF 5.0 nM KF</td>
</tr>
<tr>
<td>$K_d$</td>
<td>1.4 ± 0.2 1.6 ± 0.2</td>
<td>1.4 ± 0.2 1.0 ± 0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Uptake</th>
<th>U2-OS Cells (n = 8)</th>
<th>PK15-hENT1 Cells (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>Control 141 ± 57 50 ± 10$^a$</td>
<td>Control 57 ± 11 31 ± 6</td>
</tr>
<tr>
<td>$K_m$</td>
<td>247 ± 78 126 ± 50</td>
<td>286 ± 110 223 ± 104</td>
</tr>
</tbody>
</table>

$^a$ Significantly different from the corresponding control values (repeated measures analysis of variance with the Dunnett’s multiple comparison post test; $P < 0.05$).

**Fig. 5.** Double reciprocal plot analyses of the interaction of KF24345 with the [3H]NBMPR binding site of human erythrocyte membranes (A) and U2-OS cell membranes (B). The site specific binding of a range of concentrations of [3H]NBMPR was measured in the absence and presence of the indicated concentrations of KF24345. Data are plotted as the reciprocals of the binding of [3H]NBMPR (ordinate) against the reciprocals of the equilibrium free concentrations of [3H]NBMPR (abscissa). Membrane concentrations were adjusted to attain a similar protein level in each experiment. Each point is the mean ± S.E.M. of four (A) or five (B) experiments. $K_i$ values for KF24345 inhibition of [3H]NBMPR binding derived from these studies are 0.53 ± 0.11 and 1.8 ± 0.7 nM for the human erythrocyte and U2-OS preparations, respectively.

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*Hammond and Archer*
that seen for the other human cell membranes tested (U2-OS and erythrocyte).

These results prompted us to assess the interaction of KF24345 with the NBMPR binding site in more detail. It is well established that transporter substrates can enhance the rate of dissociation of [3H]NBMPR from its specific sites and that some inhibitors (e.g., dipyridamole and dilazep) can slow the rate of dissociation of [3H]NBMPR, relative to that produced using NBMPR analogs such as NBTGR (Jarvis et al., 1983; Hammond, 1991). KF24345 was similar to dipyridamole in that it decreased the rate of dissociation of [3H]NBMPR from human erythrocyte membranes. Thus, like dipyridamole, KF24345 is a positive allosteric modifier of [3H]NBMPR binding affinity. These dissociation studies were repeated using PK15-hENT1 membranes. Unlike that seen with erythrocyte membranes, [3H]NBMPR dissociated from the PK15-hENT1 membranes in a biphasic manner. These results, in conjunction with the KF24345 inhibition data strongly support the existence of a heterogenous population

Fig. 6. Effect of KF24345 on the $K_m$ and $V_{max}$ of [3H]nucleoside uptake by the es/ENT1 transporter of U2-OS cells and PK15-hENT1 cells. Cells were incubated with a range of concentrations of either [3H]formycin B (U2-OS cells; A) or [3H]uridine (PK15-hENT1 cells; B) for 15 s in the presence or absence of 10 nM KF24345. In parallel assays, the U2-OS cells were incubated with 100 nM NBMPR to determine the amount of uptake attributable to the ei transporter (and nonmediated uptake), and this component was subtracted from the data to assess the impact of KF24345 on the es transporter specifically. Nonspecific uptake of substrate by the PK15-hENT1 cells, which express only es-like uptake activity, was determined in parallel assays by incubation of the cells with 10 µM dipyridamole and 10 µM NBMPR, and this component was subtracted from data before the analyses represented by these figures. Each point is the mean ± S.E.M. from eight (U2-OS cells) or five (PK15-hENT1 cells) experiments conducted in duplicate.

Fig. 7. Reversibility of KF24345 and NBTGR inhibition of [3H]NBMPR binding to U2-OS cell membranes. Membranes were incubated at 22°C for 60 min in the absence (control, ■) or presence of either 100 nM KF24345 (△) or 50 nM NBTGR (○). Membranes were then washed twice by centrifugation and resuspended in PBS to assess the specific binding (picomoles per milligram of protein) of a range of concentrations of [3H]NBMPR to each preparation. Each point is the mean ± S.E.M. from four experiments conducted in duplicate.

Fig. 8. Effect of KF24345 on the rate of dissociation of [3H]NBMPR from human erythrocyte and PK15-hENT1 membranes. Membranes were incubated with 1.5 nM [3H]NBMPR for 45 min at 22°C. Dissociation of [3H]NBMPR from its binding sites was then induced by the addition of 1.4 µM NBTGR (●), dipyridamole (■), or KF24345 (○). Data are plotted as ln(B/B₀) against time, where B is site-bound [3H]NBMPR at each time after the addition of inhibitor and B₀ is site-bound [3H]NBMPR at equilibrium (time 0). The nonspecific binding component, determined as that inhibited by NBTGR after a 60-min incubation, was subtracted from each data set before analysis. Each point is the mean ± S.E.M. from five experiments conducted in duplicate.
of NBMPR binding sites in the PK15-hENT1 membranes. The slower dissociating component in the PK15-hENT1 membranes was almost identical in its characteristics to the sole NBMPR binding site in erythrocyte membranes, suggesting that this is the site typically associated with the es transporter. The identity of the more rapidly dissociating NBMPR binding component in the PK15-hENT1 membranes is unknown at this time. However, this component would likely be more susceptible to inhibition by tight binding (slow-dissociating) inhibitors such as KF24345 and might thereby explain the high affinity KF24345 inhibition component that we observed in the equilibrium binding experiments using PK15-hENT1 membranes (Fig. 4A). As discussed above, it is possible that this heterogeneity may be reflection of differential post-translational processing of some of the hENT1 proteins produced in this recombinant pig epithelial cell model and may not represent a natural physiological heterogeneity.

One of the more significant findings of this study is that KF24345 was an apparent noncompetitive inhibitor of both [3H]nucleoside uptake and [3H]NBMPR binding by the human es transporter. This makes KF24345 distinct from other blockers of the es transporter that are typically competitive inhibitors. The apparent noncompetitive type of inhibition seems due to a very slow dissociation of KF24345 from the NBMPR binding site, as shown by the inhibitor washout experiments (Fig. 7). These results are compatible with the long duration of action of the compound as an adenosine uptake inhibitor in some animal models (Noji et al., 2002c).

**Summary.** KF24345 is a potent inhibitor of both the es and ei transporter of mammalian cells. It is as effective as the prototypical inhibitor NBMPR at inhibiting the es transporter in human cells, but it differs from NBMPR in that it also blocks the ei transporter with an IC50 in the nanomolar range. KF24345 shows less species heterogeneity in its es affinity than do other inhibitors such as dipyridamole and may thus prove to be a useful inhibitor in a range of animal models. The tight binding characteristics of KF24345 interaction with the es transporter, resulting in apparent noncompetitive inhibition kinetics, should be of value in future studies on the delineation of the inhibitor binding domains of the ENT protein. The long-lasting adenosine uptake blockade resulting from these binding kinetics will also aid in its use as an in vivo inhibitor of nucleoside transport in studies assessing the effects of adenosine on biological systems.

**Acknowledgments**

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