Inhibition of Nucleoside Transport by Protein Kinase Inhibitors

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ABSTRACT

Recently we reported that the pyridinylimidazole class of p38 mitogen-activated protein (MAP) kinase inhibitors potently inhibited the facilitated transport of nucleosides and nucleoside analogs in K562 cells. These compounds competed with the binding of nitrobenzylthioinosine (NBMPR) to K562 cells, consistent with inhibition of the NBMPR-sensitive equilibrative transporter (ENT1). In this study we examined a large number of additional protein kinase inhibitors for their effects on nucleoside transport. We find that incubation of K562 cells with tyrosine kinase inhibitors (AG825, AG1517, AG1478, STI-571), protein kinase C (PKC) inhibitors (staurosporine, GF 109203X, R0 31-8220, arcyriarubin A), cyclin-dependent kinase inhibitors (roscovitine, olomoucine, indirubin-3′-monoxime), or rapamycin resulted in a dose-dependent reduction of intracellular uptake of [^{3}H]uridine. In contrast, neither the MAP kinase kinase inhibitors (U0126, PD 98059) nor the phosphatidyl inositol-3 kinase inhibitors (wortmannin, LY 294002) affected this process. Furthermore, both transient uptake and prolonged [^{3}H]thymidine incorporation in K562 cells were inhibited by protein kinase inhibitors, inactive analogs of kinase inhibitors (R0 31-6045, SB202474), and NBMPR, independently of effects on cell proliferation as determined by MTT assay. These studies demonstrate that a wide variety of protein kinase inhibitors affect nucleoside uptake through selective inhibition of nucleoside transporters, independently of kinase inhibition.

The design of small molecule inhibitors of protein kinases is currently an area of intense interest. Spurred on by clinical promises such as those obtained recently with Gleevec (STI-571) (Shah and Sawyers, 2001), the drive to find additional, selective inhibitors of protein kinases for the treatment of cancer, arthritis, auto immune disorders or other pathologies continues. Recent studies have investigated the efficacy by which protein kinase inhibitors (PKIs) may function as modulators of currently available cytotoxic agents including immunosuppressive drugs, anticancer, and antiviral compounds (Swannie and Kaye, 2002). Several studies suggest that inhibitors of protein kinase C (PKC) or protein tyrosine kinases modulate the sensitivity of tumor cells to conventional chemotherapeutic drugs (Swannie and Kaye, 2002). These include the PKC inhibitors CGP 41251 (Ganeshaguru et al., 2002), Ro 32-2241 (Merritt et al., 1999), ISIS 3521 (Swannie and Kaye, 2002), and GF 109203X (Gekeler et al., 1995); tyrphostins family AG1571 (SU-5271) (Ben-Bassat, 2001), tyrphostin AG825 (Tsai et al., 1996), or genistein

ABBREVIATIONS: PKI, protein kinase inhibitor; PKC, protein kinase C; dFdC, gemcitabine; Ara-C, cytarabine; CdA, cladribine; F-ara-A, fludarabine; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; NBMPR, nitrobenzylmercaptopurine ribonucleoside; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; DMSO, dimethyl sulfoxide; PTK, protein tyrosine kinase; TOR, target-of-rapamycin; EGFR, endothelial growth factor receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridinyl)imidazole; SB203580-iodo, 4-(3-iodophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SB220025, 5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole; SB202474, 4-(ethyl)-2-(4-methoxyphenyl)-5-(4-pyridyl)-1H-imidazole; PD153035 (AG1517), 4-(3-bromophenyl)amino-6,7-dimethoxyquinazoline; AG 1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; WHI-P154, 4-[(3-bromo-4-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline; WHI-P131, 4-(4-hydroxyphenyl)amino-6,7-dimethoxyquinazoline; WHI-P258, 4-phenylamino-6,7-dimethoxyquinazoline; WHI-P180, 4-(3-hydroxyphenyl)amino-6,7-dimethoxyquinazoline; WHI-P97, 4-(3,5-dibromo-4-hydroxyphenyl)amino-6,7-dimethoxyquinazoline; AG18, α-cyano-(3,4-dihydroxy)cinnamonitrile; AG490, α-cyano-(3,4-dihydroxy)-N-benzylcinnamide; AG 1879 (PP2), 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; H89 dihydrochloride, N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide 2HCl; ZM336372, N-[5-(3-dimethylaminobenzamido)-2-methylphenyl]-4-hydroxybenzamide; KN93, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; LY 294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; arcyriarubin A, 2,3-bis(1H-indol-3-yl)maleimide; Ro 31-8220, 3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide; Ro 32-0432, 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-α]indol-3-yl}-3-(1-methyl-1H-indol-3-yl)maleimide HCl; Ro 31-6045, 2,3-bis(1H-indol-3-yl)-N-methylmaleimide; GF 109203X, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide; olomoucine, 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine; roscovitine, 2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine.

(Tanos et al., 2002). As a result, increasing attention has been devoted to identifying additional targets for PKIs that may influence the biological responses to these compounds.

Nucleoside analogs that interfere with nucleotide metabolism and DNA replication are widely used in anticancer or antiviral therapies (Pastor-Anglada et al., 1998). Specifically pyrimidine nucleoside derivatives such as gemcitabine (dFdC) and cytarabine (Ara-C), pyrimidine derivatives (bromodeoxyuridine and iododeoxyuridine), and the purine derivatives cladribine (CdA) and fludarabine (F-ara-A) are currently used as chemotherapeutic agents (Pastor-Anglada et al., 1998; Galmarini et al., 2001). The clinical efficacy of these compounds is highly dependent on the rates of uptake across the plasma membrane, which is mediated by specific nucleoside transporters (Pastor-Anglada et al., 1998).

The recent cloning of the genes for the nucleoside transporters has resulted in the identification of many of the specific transporters responsible for these events (Pastor-Anglada et al., 2001). The concentrative nucleoside transporters (CNTs) transport nucleosides and nucleoside analogs in a sodium-dependent manner (Cass et al., 1999). However, the most widely expressed nucleoside transporter subtype in mammalian cells or tissues is the equilibrative nucleoside transporters (ENTs), which transport nucleosides in a sodium-independent manner. These transporters can be further defined by their differential sensitivity (es/ENT1) or insensitivity (ei/ENT2) to the inhibitor, nitrobenzylthioinosine (NBMPR) (Cass et al., 1999). The es/ENT1 equilibrative transporters are also sensitive to inhibition by the cardioprotective agents dilazep, draflazine, and dipyridamole (Baldwin et al., 1999). Although the es/ENT1 transporters have a similar affinity for NBMPR, significant species differences have been observed in the sensitivity of the es/ENT1 transporters to other structurally diverse inhibitors such as dipyridamole and draflazine (Hammond, 2000). The finding that these structurally unrelated inhibitors show differential affinity for the es/ENT1 transporter from different species and that dipyridamole is capable of competing the binding of [³H]NBMPR in a variety of experimental models (Hammond, 2000) suggests that dipyridamole and NBMPR have distinct but overlapping binding sites in the nucleoside transporters (Hammond, 2000).

The human erythroleukemia K562 cell line was isolated and characterized from a patient with chronic myelogeneous leukemia in blast crisis, and expresses the p210 Bcr-Abl fusion protein (Ramakrishnan and Rosenberg, 1989). In this cell line, approximately 80 to 90% of total nucleoside transport activity occurs by equilibrative NBMPR-sensitive (es) transport whereas the remainder occurs by an NBMPR-insensitive (ei) transport process (Boleti et al., 1997). Using this cell line as a model system, the effects of p38 MAPK inhibitors on the equilibrative transport process were previously evaluated (Huang et al., 2002).

In our current study, we have investigated a group of structurally distinct PKIs for their effects on nucleoside transport. We report here that a wide number of these compounds inhibit the uptake of both [³H]uridine and [³H]thymidine by specific inhibition of the ENT1 nucleoside transporter. Thus these studies further demonstrate that the equilibrative transporters are targets for the cellular effects of PKIs.

Materials and Methods

Cell Cultures and Reagents. Human erythroleukemia K562 cells were cultured as described earlier (Huang et al., 2002). Uridine ([5,6-³H], 35–50 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). [³H]NBMPR (22.5 Ci/mmol) and [5-³H]cytosine-β-D-arabinofuranoside ([5-³H]Ara-C, 15–30 Ci/mmol), and [*methyl*-³H]thymidine (20.0 Ci/mmol) were from Moravek Biochemicals (Brea, CA). Uridine, thymidine, adenosine, and NBMPR (nitrobenzylthioinosine, 6-[(4-nitrobenzyl)thio]-9-(β-D-ribofuranosyl) purine) were obtained from Sigma-Aldrich (St Louis, MO). SB202474, SB203580, SB203580-iodo, SB220025, Ro 31-6045, arcyriarubin A, GF 109203X, Ro 31-8220, Ro 32-0432, staurosporine, AG825, AG18, AG1478, AG1517 (PD153035), WHI-154, WHI-P97, WHI-180, WHI-P258, vascular endothelial growth factor (VEGF) tyrosine kinase inhibitor, AG490, AG1879 (PP2), rapamycin, Raf-1 inhibitor 1, H-89, KN93, genistein, wortmannin, LY-294002, and ZM336372 were purchased from Calbiochem-Novabiochem (La Jolla, CA). U0126 and PD 98059 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). STI-571 was provided by E. Buchdunger (Novartis, Basel, Switzerland).

Uptake Assays of [³H]Uridine and [³H]Thymidine. The assays of [³H]uridine uptake were conducted as described previously in sodium-containing buffer (20 mM Tris/HCl, 3 mM K₂HPO₄, 1 mM MgCl₂ · 6H₂O, 2 mM CaCl₂, 5 mM glucose, and 130 mM NaCl, pH 7.4) (Huang et al., 2002). Briefly, 5×10^5 K562 cells/sample were washed once with transport buffer and then resuspended in 400 μl of transport buffer. After preincubation with SB analogs, NBMPR, or DMSO for 15 min, uptake assays were started by adding equal volume of transport buffer containing 10 μM ³H-labeled uridine (4 μCi/ml) or ³H-labeled thymidine (10 μCi/ml) plus inhibitors or DMSO. Uptake assays were stopped at 60 or 30 s, respectively, by five rapid washes with ice-cold transport buffer containing 1 mM unlabeled competing uridine or thymidine. The cell pellets were lysed in 10% SDS before quantification of radioactivity.

Measurement of [³H]Thymidine Incorporation (Gotoh et al., 2002). For [³H]Thymidine incorporation, exponentially growing K562 cells were plated in 1-ml aliquots of growth medium into 24-well plates at 4×10^4 cells per well, respectively, and then treated with various PKIs or vehicle DMSO control. After 24 h of treatment, [³H]thymidine was added at a final concentration of 1 μCi/ml for an additional 4 h. At harvest, cells were washed twice with ice-cold phosphate-buffered saline, and precipitated by 5% trichloroacetic acid at 4°C. The pellets were washed with ice-cold 95% ethanol, dried at room temperature, and then dissolved in 200 μl of 1 M NaOH for 30 min before quantification of radioactivity. Triplicate wells were analyzed for each treatment.

Measurement of Cell Proliferation by MTT Assay. The effects of PKI's on proliferation of K562 cells were determined using the modified MTT assay method (Carmichael et al., 1987). Exponentially growing K562 cells were plated in 1-ml aliquots of growth medium into 24-well plates at 4×10^4 cells per well, respectively, and then incubated with various PKIs or vehicle alone. After 24 or 48 h of incubation, cytotoxicity assays were performed by the modified MTT method. Triplicate wells were analyzed for each treatment.

Results

A Structurally Diverse Group of Protein Kinase Inhibitors Prevent Nucleoside Transport in K562 Cells. To extend our studies on the effects of PKIs on nucleoside transport, we tested a series of compounds of different specificities. Incubation of K562 cells with selective inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase (Erb B1), AG1517 (also known as PD 153035) (Levitzki and Gazit, 1995), AG1478 (Levitzki and Gazit, 1995), the Erb B2 tyrosine kinase (AG825) (Tsai et al., 1996), significantly in-

hibited the uptake of [³H]uridine in these cells (Fig. 1). Similarly, potent inhibition of uridine transport was observed with the Bcr-Abl tyrosine kinase inhibitor, STI-571 (Shah and Sawyers, 2001). At 10 μM, these compounds inhibited greater than 50% of [³H]uridine transport in these cells, whereas other related compounds (AG18, AG1879, AG490, or the general tyrosine kinase inhibitor, genistein) were less effective at this concentration.

Analysis of a number of serine/threonine kinase inhibitors showed that the broad specificity PKC inhibitor (staurosporine), the Raf1 kinase inhibitor (Lackey et al., 2000), cyclin-dependent kinase inhibitors (roscovitine, olomoucine, and indirubin-3′-monoxime) (Buolamwini, 2000), and the target-of-rapamycin (TOR) kinase inhibitor (rapamycin) also strongly inhibited nucleoside transport (Fig. 1). Partial inhibition was observed with the protein kinase A inhibitor H-89, the CAMKII selective inhibitor KN93, and the Raf kinase inhibitor ZM336372. By contrast, neither the MEK1 inhibitors (PD 98059, U0126) nor the phosphatidyl inositol-3 kinase inhibitors (wortmannin, LY-294002) significantly inhibited the uptake of [³H]uridine in these cells when assayed at 10 μM, thus demonstrating the differential effects of these compounds on nucleoside transport.

Inhibition of [³H]Uridine Transport by a Related Class of Protein Tyrosine Kinase Inhibitors. Because our results showed that the tyrphostin class of protein tyrosine kinase inhibitors (PTKs) inhibited nucleoside transport, we further examined the structure-activity relationship of these compounds. Specifically, the quinazoline tyrphostin AG1478 and the corresponding bromo derivative AG1517 (PD 153035), two selective inhibitors of EGFR kinase activity (Levitzki and Gazit, 1995), were compared to other dime-

thoxyquinazoline derivatives for their inhibitory effects on the uptake of [³H]uridine in K562 cells (Fig. 2A). As shown in Fig. 2B, WHI-P154, the 4′-hydroxyphenyl derivative of AG1517 and inhibitor of JAK3 tyrosine kinase (Sudbeck et al., 1999), exhibited similar inhibitory effects on [³H]uridine uptake to that observed with AG1517 and AG1478. WHI-P258, an inactive analog of the JAK3 inhibitor (Sudbeck et al., 1999), the VEGF tyrosine kinase inhibitor (Hennequin et al., 1999), or WHI-P97 (Sudbeck et al., 1999) showed a reduced ability to inhibit nucleoside transport in these cells and correlated with increasing substitutions on the 4′-phenyl group or the loss of an electronegative group at the 3′ position of this ring.

Comparison of AG1517 and AG1478 to the structurally distinct compound AG825 (Fig. 2C) demonstrated that these compounds suppressed [³H]uridine uptake with a similar IC_{50} 0.9, 1.0, and 1.1 μM, respectively (Fig. 2D). By contrast, the other tyrphostins, AG 18 (a broad-spectrum kinase inhibitor), AG490 and AG 1879 (Fig. 2C), were not as effective at inhibiting transport as shown in Fig. 1.

PKC Inhibitors are Potent Inhibitors of Nucleoside Transport. Since our data in Fig. 1 demonstrated that the PKC inhibitor staurosporine prevented nucleoside transport (Fig. 1), we examined a series of related PKC inhibitors including GF 109203X, Ro 31-8220, and arcyriarubin A (Fig. 3A). Incubation of K562 cells with these compounds at concentrations from 0.1 to 10 μM induced a dose-dependent inhibition of [³H]uridine uptake with an IC_{50} of 1.7, 0.8, and 1.7 μM, respectively (Fig. 3B). Interestingly, Ro 32-0432, another derivative of staurosporine and selective inhibitor of PKC (Birchall et al., 1994), did not inhibit nucleoside transport at concentrations up to 10
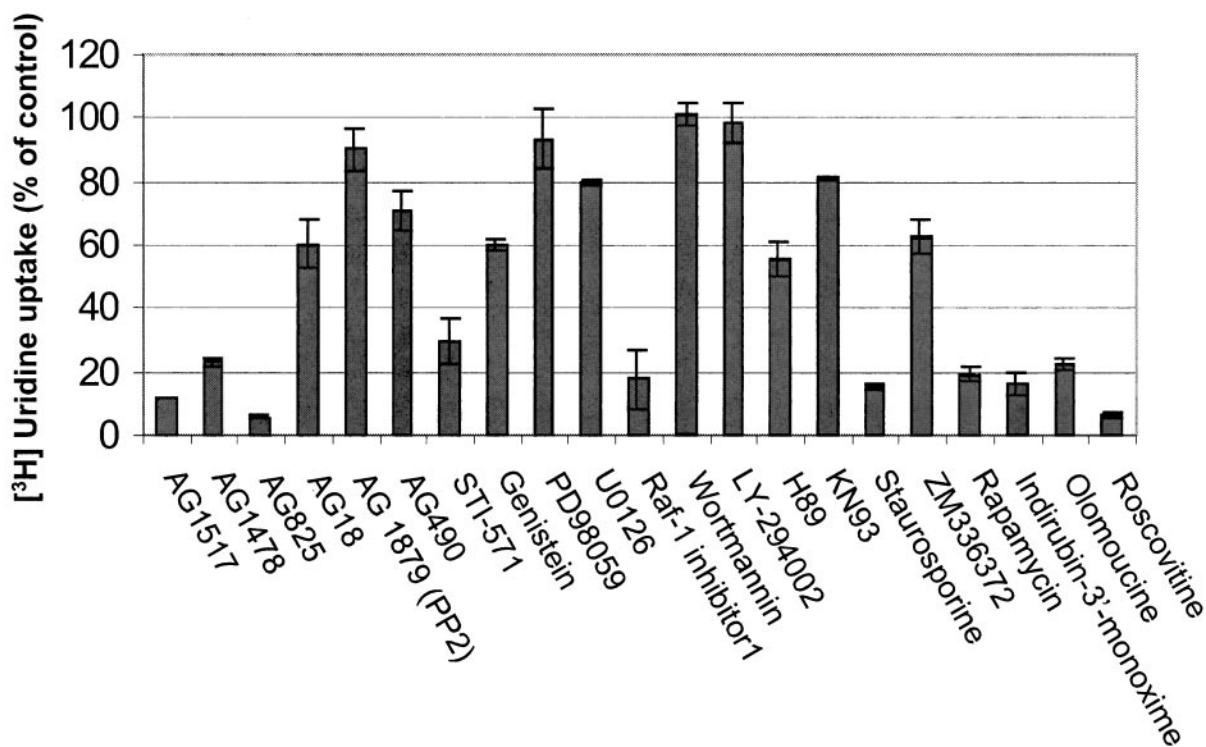
Fig. 1. A large number of PKIs block uridine nucleoside transport. K562 cells were preincubated with individual PKIs (10 μM) or DMSO for 15 min. 5 μM [³H]uridine was then added over a 1-min period, and the uptake was measured as described under *Materials and Methods*. Data are shown as percentage of control uptake. Each point represents the mean $\pm$ S.D. from $n = 4$ samples conducted in duplicate.
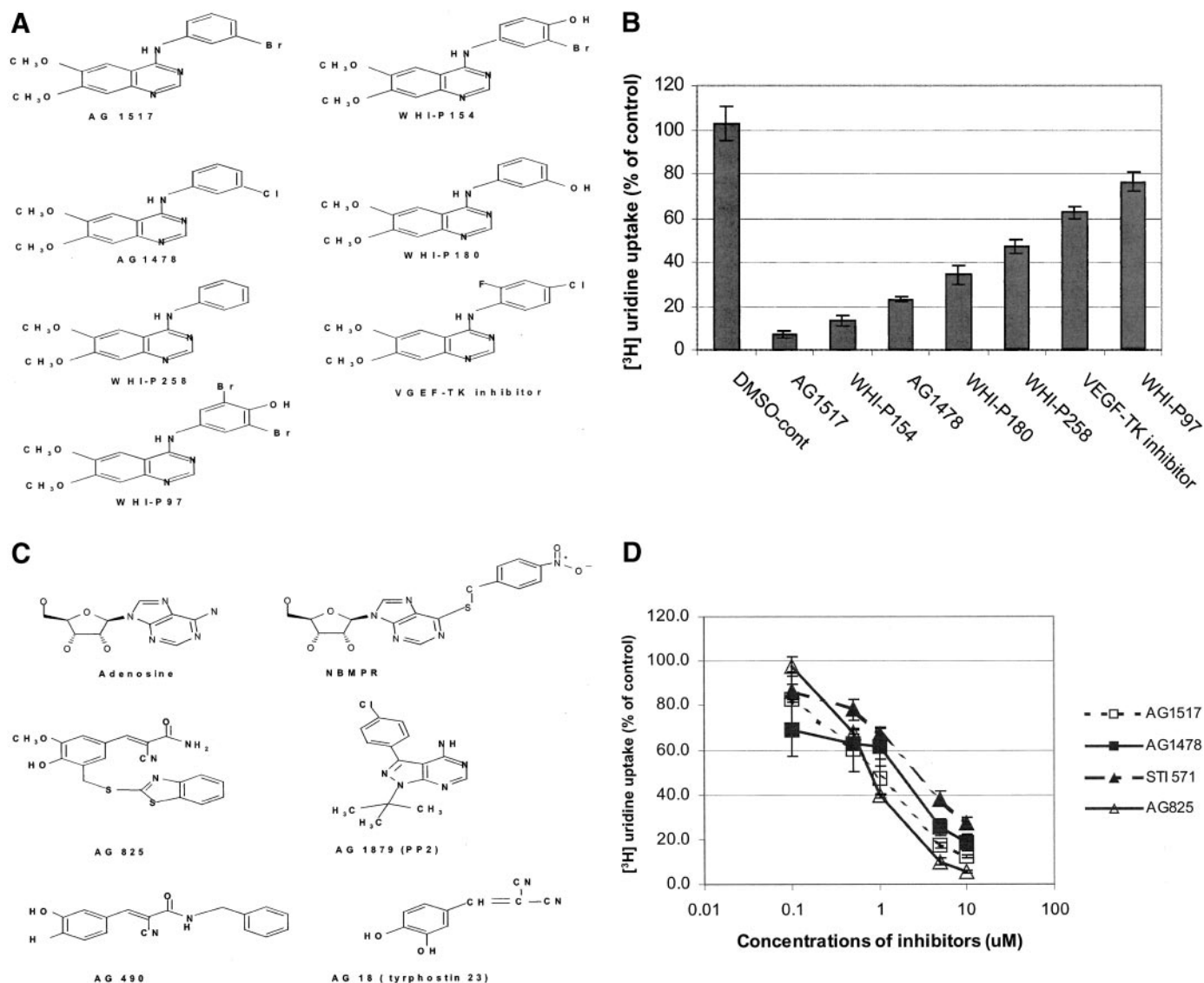
Fig. 2. The structure-activity relationship of dimethoxyquinazoline and tyrphostin analogs in regulating nucleoside transport activity. A, chemical structure of dimethoxyquinazoline analogs shown in decreasing order (left to right, top to bottom) in inhibitory potency of nucleoside transport. Variations in the 4′-phenyl ring are shown. B, the dimethoxyquinazoline analogs were tested for their capacity to inhibit the uptake of 5 μM [^{3}H]uridine in K562 cells at a final concentration of 10 μM. Data are shown as percentage of DMSO (vehicle) binding, and each point represents the mean $\pm$ S.D. from $n = 4$ samples conducted in duplicate. C, chemical structure of adenosine, NBMPR, and several tyrphostins. D, the IC$_{50}$ of AG1517, AG1478, and AG825 on uridine uptake was determined by incubation with 5 μM [^{3}H]uridine in the presence of 0.1 to 10 μM individual compounds or DMSO control for 1 min. Data are shown as percentage of control binding, and each point represents the mean $\pm$ S.D. from $n = 4$ samples conducted in duplicate.

μM. In comparison, Ro 31-6045, a staurosporine analog that does not inhibit PKC (Davis et al., 1992), showed the most potent inhibitory effects on [^{3}H]uridine transport with an IC$_{50}$ of 0.06 μM suggesting that the effects of these compounds on nucleoside transport were independent of PKC inhibition.

Effects of p38 MAPK Inhibitors and Other PKIs on [^{3}H]Thymidine Uptake in K562 Cells. The incorporation of ^{3}H-labeled thymidine into DNA is a widely used method to evaluate the rate of cell proliferation. Because our previous study demonstrated that the SB class of p38 MAPK inhibitors potently blocked the transport of [^{3}H]uridine, we examined whether similar effects were observed with [^{3}H]thymidine uptake. Incubation of K562 cells with concentrations from 0.1 to 10 μM demonstrated that SB203580-iodo, SB203580, and SB202474 inhibited the

uptake of [^{3}H]thymidine of K562 cells in a dose-dependent manner, whereas SB220025 was largely without effect (Fig. 4A). Since SB202474 does not inhibit p38 MAPK (Lee et al., 1994) whereas SB220025 does (Wang et al., 1998), these results indicated that the effects of these compounds on thymidine uptake occurred independently of p38 MAPK inhibition and were similar to that observed earlier with uridine transport (Huang et al., 2002). Like the p38 MAPK inhibitors, a series of other PKIs (STI-571, AG825, AG1517, AG1478) (Fig. 4B) or Ro 31-8220, Ro 31-6045, GF 109203X, or arcyriarubin A (Fig. 4C) also showed potent inhibitory effects on the transport of [^{3}H]thymidine into these cells (Fig. 4B). By contrast, Ro 32-0432, did not inhibit the uptake of [^{3}H]thymidine into these cells (Fig. 4C), consistent with the lack of effects of this compound on [^{3}H]uridine transport (Fig. 3B).
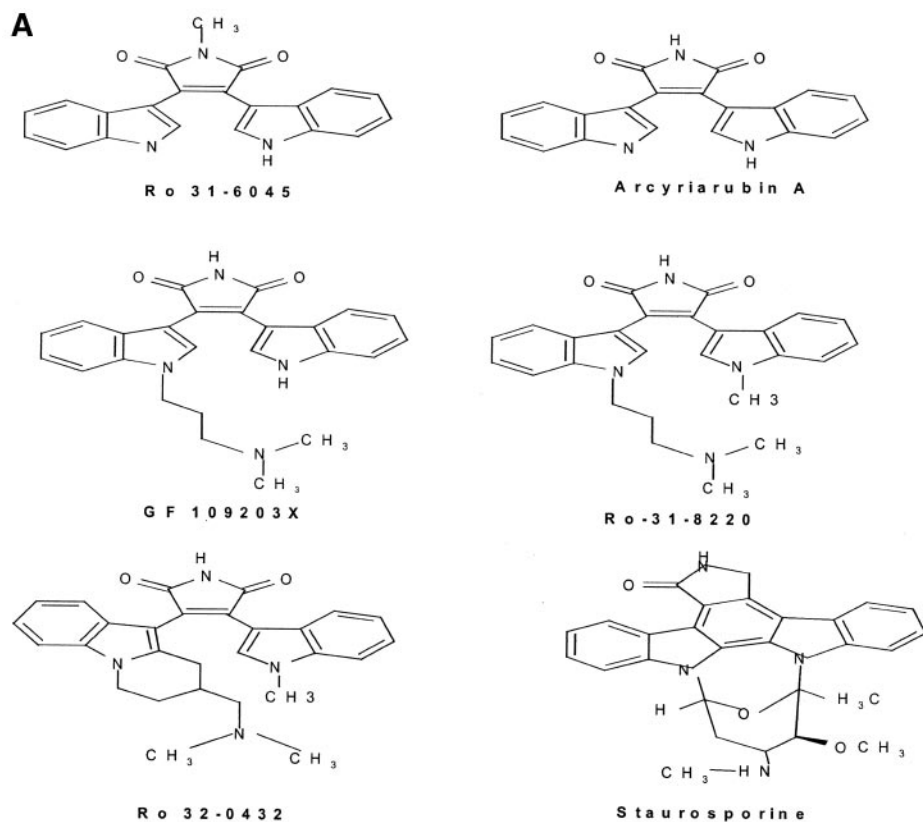
Fig. 3. The structure-activity relationship of staurosporine analogs in regulating [³H]uridine uptake. A, the staurosporine analogs used were divided into two groups based on their structure as the indolocarbazoles with an intact aromatic ring structure (staurosporine) and the bisindolylmaleimides with an opened central aromatic ring (GF 109203X, Ro 31-8220, Ro 32-0432, arcyriarubin A, and Ro 31-6045). B, the staurosporine analogs were tested for their capacity to inhibit the uptake of 5 μM [³H]uridine in K562 cells. The IC_{50} of those compounds on uridine uptake was determined by incubation with 5 μM [³H]uridine in the presence of 0.1 to 10 μM individual compounds or DMSO control for 1 min. Data are shown as percentage of control binding. Each point represents the mean $\pm$ S.D. from $n = 4$ samples conducted in duplicate.
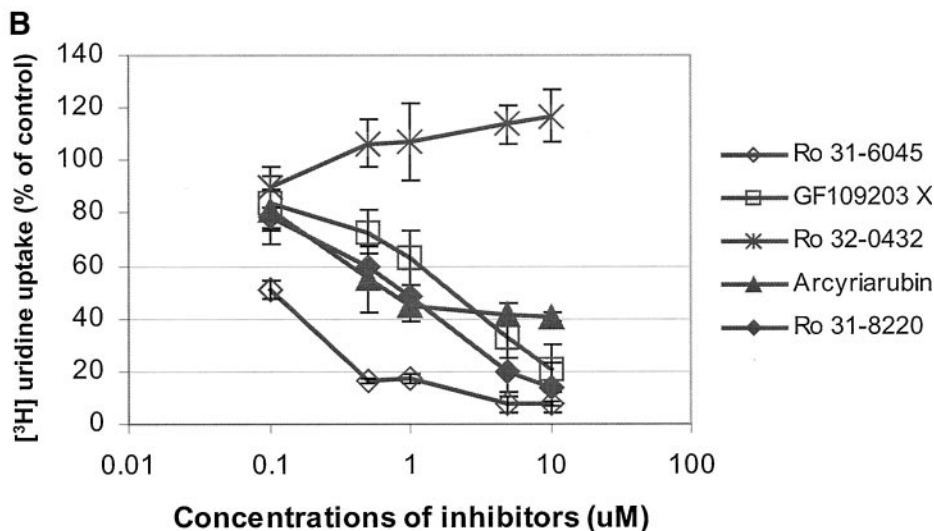
The Effects of PKIs on [³H]Thymidine Incorporation Does Not Correlate with the Effects of These Compounds on Cell Proliferation. Because our results suggested that PKIs inhibited the transport of [³H]thymidine, we examined whether the [³H]thymidine incorporation into DNA was similarly affected. Consistent with the effects of these compounds on either [³H]uridine or [³H]thymidine uptake, the incorporation of ³H-labeled thymidine into DNA was inhibited by treatment of K562 cells with the tyrphostins AG825, AG1517, AG1478, bisindolylmaleimides GF 109203X, Ro 31-6045, arcyriarubin A, Ro 31-8220, STI-571, and NBMPR, a potent ENT1 inhibitor for 24 h (Fig. 5A and data not shown). However, comparing the effects of these compounds on the MTT cell proliferation assay demonstrated important differences. Although significant inhibition of ³H-labeled thymidine into DNA was observed, only Ro 31-8220 and STI-571 significantly inhibited cell proliferation at 24 h. Instead, when assayed at concentrations up to 10 μM, NBMPR, Ro 31-6045, and the other PKIs showed little or no effect on cell proliferation (Fig. 5A and data not shown) though Ro 31-6045 showed an inhibitory effect on cell proliferation at concentrations higher than 5 μM after 48 h of treatment. Thus these results demonstrate that the effects of NBMPR, Ro 31-6045, and some PKIs on [³H]thymidine uptake and incorporation can be dissociated from the effects on cell proliferation.
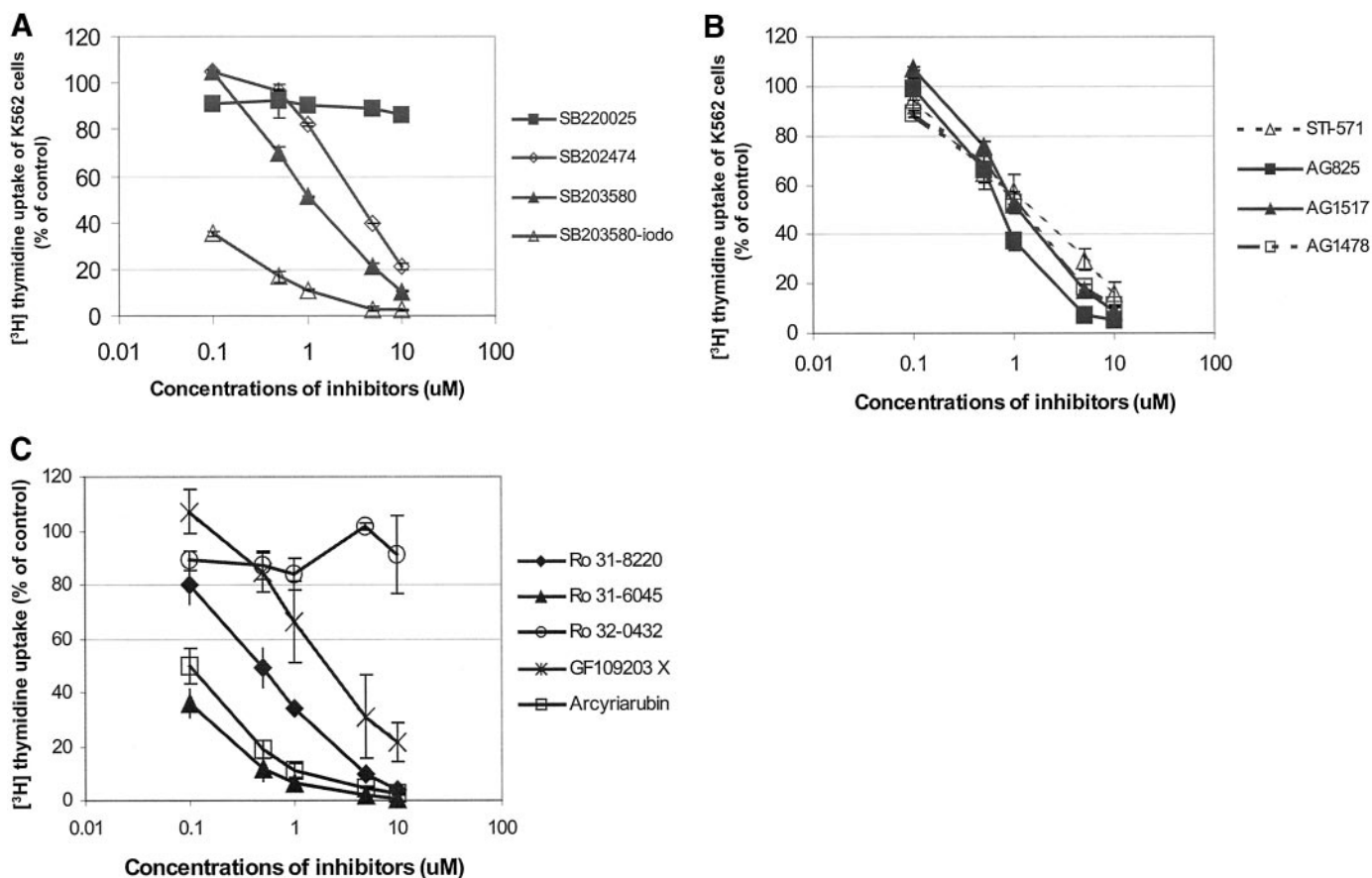
Fig. 4. Parallel inhibition of [³H]thymidine uptake by p38 MAPK, protein tyrosine kinase, and PKC inhibitors. Inhibition of [³H]thymidine uptake by p38 MAPK inhibitors (A), PTK inhibitors (B), and PKC inhibitors (C) was conducted as described under *Materials and Methods*. K562 cells were preincubated in the presence of individual compounds at a range of 0.1 to 10 μM or DMSO control for 15 min, and then thymidine uptake was determined by incubation of K562 cells with [³H]thymidine (5 μCi/ml) in the presence of 0.1 to 10 μM individual compounds or DMSO control for 1 min. Data are shown as percentage of control binding. Each point represents the mean ± S.D. from n = 4 samples conducted in duplicate.

Discussion

Uncontrolled signaling from serine/threonine kinases (i.e., MAPK) or PTKs has been linked to proliferative and inflammatory diseases (Ben-Bassat, 2001). Numerous PKIs have been designed to compete with ATP binding to the catalytic cleft of protein kinases to achieve selective inhibition of these enzymes for clinical or experimental purposes. The quinazolines AG1478, AG1517 (Arteaga et al., 1997), and IRESSA (Lichtner et al., 2001), specific inhibitors of the EGFR kinase, are believed to prevent kinase function by sequestering receptor into inactive dimers through interaction with the receptor ATP binding sites, thereby disrupting receptor signaling (Arteaga et al., 1997). The results of the current study now extend our previous observations that the pyridinylimidazole class of PKIs potently inhibit the facilitated uptake of nucleosides into mammalian cells. We demonstrate that a large number of widely used inhibitors of receptor tyrosine kinases, PKC, cyclin-dependent kinases, Bcr-Abl, or TOR significantly inhibit both the uptake and the incorporation of nucleosides. Like that observed with the SB203580 class of p38 MAPK inhibitors (Huang et al., 2002), we find that the effects of PKIs on nucleoside transport are structure-dependent and independent of kinase inhibition. Moreover, a number of kinase inhibitors used at concentrations known to inhibit their respective kinases (i.e., 10 μM U0126, PD

98059, wortmannin, LY 294002) had little or no apparent effect on nucleoside transport, therefore highlighting the specificity of these effects.

Despite the fact that we found that a large number of structurally diverse PKIs inhibited transport, insight into the structure-function relationship for these compounds is emerging. Like NBMPR, a well known ENT1 inhibitor, the kinase inhibitors that are effective in inhibiting nucleoside transport are structural purine or pyrimidine analogs or structurally related compounds. Analysis of the tyrphostins, tyrosine kinase inhibitors, or the bismaleimide PKC inhibitors has allowed comparison of the structural requirements for these compounds. Within the tyrphostins, the most potent compound was AG1517. Increasing the number of substitutions on the 4′-phenyl group or the loss of a strong electronegative group (i.e., Br, Cl) at the 3′ position of this ring significantly reduced the efficacy by which these compounds affected nucleoside transport. By contrast, WHI-P154, the 4′-hydroxyphenyl derivative of AG1517 exhibited similar inhibition of [³H]uridine uptake to AG1517 and AG1478, though it is reported to have reduced inhibitory effects on the EGFR kinase itself (Sudbeck et al., 1999). Although tyrphostin AG825 remarkably suppressed the uptake of [³H]uridine, the structurally unrelated tyrosine kinase inhibitor tyrphostin AG 18 and AG 490 only slightly inhibited uridine transport.
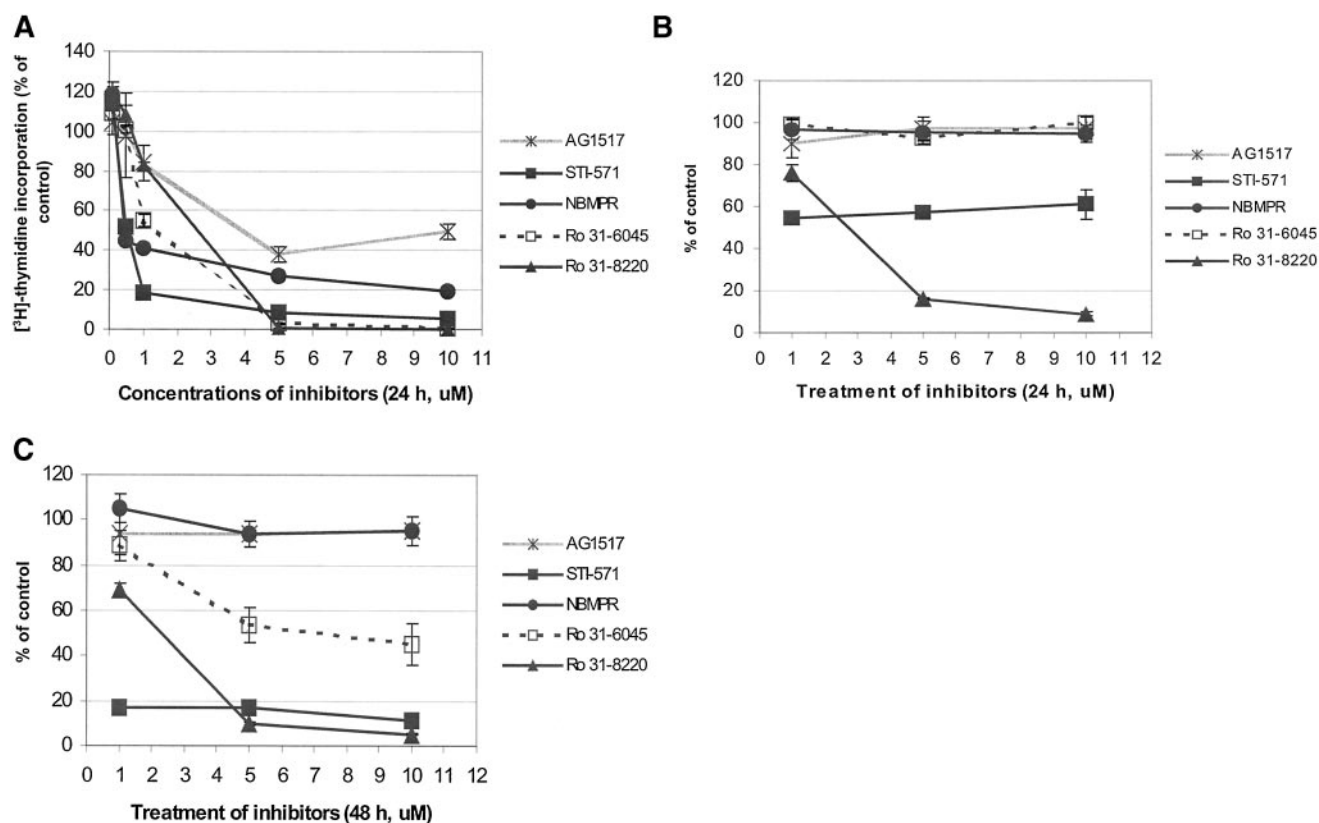
Fig. 5. Effects of PKIs on incorporation of [³H]thymidine into DNA and cell proliferation. A, effect of PKIs on proliferation of K562 cells. K562 cells were seeded in 24-well plates at a density of 4×10^4 cells/ml, and inhibitors at concentrations indicated or vehicle control were added. After 24 h of incubation with inhibitors, the cells were pulsed with 1 μCi/ml [³H]thymidine in RPMI 1640 medium for an additional 4 h. Data are shown as percentage of control. Each point represents the mean ± S.D. from $n = 4$ samples conducted in duplicate. B and C, K562 cells were analyzed for uptake of MTT in the presence or absence of various PKIs for 24 and 48 h. Data are plotted as the concentrations of PKIs versus inhibition of proliferation. Each data point represents a mean across three identical wells from one representative experiment.

Similarly, the PKC class of inhibitors has revealed important structural requirements for the inhibition of nucleoside transport. Staurosporine and related analogs inhibit PKC by competing with ATP binding to the catalytic domain of PKC (Herbert et al., 1990). Although staurosporine is considered a nonselective PKI, a new generation of bisindolylmaleimide derivatives with an opened central aromatic ring has been shown to have significantly improved selectivity for PKC (Mahata et al., 2002). Three of the bisindolylmaleimide derivatives (GF 109203X, arcyriarubin A, and Ro 31-8220) with relatively similar inhibitory potency (Mahata et al., 2002) showed similar effects on nucleoside transport. Of the bisindoylmaleimide compounds analyzed, only Ro 32-0432, another potent PKC inhibitor, did not inhibit nucleoside transport in these cells. Structurally, this may be related to the substitution of the (dimethylamino)methyl tetrahydropyridol group on the indole ring of this compound; addition of this bulky group may compromise the ability of Ro 32-0432 to inhibit nucleoside transport. By contrast, Ro 31-6045 (bisindolylmaleimide V), an inactive analog, was the most potent inhibitor of [³H]uridine uptake within those bisindolylmaleimides tested. Methylation of the amino group within the maleimide ring in bisindolylmaleimide V (Ro 31-6045) abolishes the inhibitory activity on PKC; however, neither methylation of this group nor addition of the cationic tail at the indole nitrogen affected the inhibition of nucleoside transport. Taken together, these results demonstrate that the effects of these compounds on nucleoside transport are independent of PKC inhibition.

The observation that both the uptake of [³H]uridine and [³H]thymidine were blocked by PKIs may be expected since both nucleosides have been shown to be transported by the equilibrative transporter (ENT1) (Ward et al., 2000). In addition to the short-term transport, the long-term incorporation of [³H]thymidine into DNA, a common assay for cell proliferation, was also prevented. However, the inhibitory effects of these compounds did not correlate with the inhibition of cell proliferation by MTT analysis. This was especially clear with the transport inhibitor NBMPR, suggesting a reason for caution when using [³H]thymidine incorporation to evaluate cell proliferation.

The implications of these studies for the application of PKIs in clinical studies should be mentioned. Our finding that a range of PKIs including STI-571 are also inhibitors of nucleoside transport raises the possibility of antagonism between PKIs and compounds used as anticancer or immunosuppressive agents. Cellular uptake of nucleoside analogs such as hydroxyurea, Ara-C, and gemcitabine occurs by equilibrative nucleoside transport (Gourdeau et al., 2001; Valdes et al., 2002) and increasing evidence has shown that the combination of PKIs with anti-leukemia drugs can produce additive, synergistic, or antagonistic effects in combination chemotherapy. For instance, the combination of STI-571 with Ara-C in several leukemia cell lines including K562 cells resulted in synergistic effects (Raina et al., 2002), whereas the combination of STI-571 and hydroxyurea (Thiesing et al., 2000) or methotrexate (Kano et al., 2001) demonstrated antagonistic effects. This prediction was coinci-

dent with a recent finding that inhibition of c-abl with STI-571 attenuated the Ara-C-dependent activation of the stress-activated protein kinase activation and apoptosis (Raina et al., 2002). BIBW22BS, a dipyridamole derivative and a highly efficient inhibitor of equilibrative nucleoside transport, inhibited the effects of gemcitabine in a variety of cancer cell lines up to 100-fold (Jansen et al., 1995). Moreover, a nucleoside transport-deficient variant of CCRF-CEM leukemia cell line (CEM/ARAC8C) exhibited high levels of resistance to Ara-C (1105-fold) and gemcitabine (432-fold) (Gourdeau et al., 2001).

Finally, activated immune cells and most solid tumors show a higher number of NBMPR (NBTI) binding sites than their normal counterparts (Goh et al., 1995), indicating increased capability for nucleoside transport. These cells may have increased dependence on nucleoside salvage for nucleic acid synthesis. Thus, modifying the activity of nucleoside transporters by PKIs may offer an attractive approach to target both protein kinases and nucleoside transport and potentially affect the outcome of immunosuppressive or anti-cancer therapies. In this regard, rapamycin, a clinically important immunosuppressive agent, also strongly inhibited the uptake of nucleosides in this study. In theory, inhibition of plasma nucleoside salvage rescue by protein kinase inhibitors such as rapamycin could potentially enhance chemotherapy of inhibitors of de novo purine or pyrimidine synthesis (e.g., leflunomide, mycophenolic acid, and methotrexate). Whether part of the clinical efficacy of these compounds is derived from inhibition of nucleoside transport remains to be determined.

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