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Translocator Protein (TSPO) Ligand−Ara-C (Cytarabine) Conjugates as a Strategy To Deliver Antineoplastic Drugs and To Enhance Drug Clinical Potential

Nunzio Denora,[†] Valentino Laquintana,[†] Adriana Trapani,[†] Angela Lopedota,[†] Andrea Latrofa,[†] James M. Gallo,[‡] and Giuseppe Trapani*[,†]

Dipartimento Farmaco-Chimico, Facoltà di Farmacia, Università degli Studi di Bari, Via Orabona 4, 70125 Bari, Italy, and Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029, United States

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Abstract: The aim of this work was to evaluate TSPO ligand−Ara-C conjugation as an approach for the selective delivery of the antineoplastic agent to brain tumors as well as for overcome P-gp resistance induction observed for the majority of cytotoxic agents, enhancing the drug clinical potential. To this end, the novel *N*-imidazopyridinacetyl−Ara-C conjugates **3a**−**c**, **10** and **15** have been prepared and evaluated for their cytotoxicity against glioma cell lines. In contrast to that observed for **3a**−**c** and **10**, the conjugate **15** resulted stable in both phosphate buffer and physiological medium. In all cases, the release of free Ara-C from hydrolyzed conjugates was checked by HPLC and ESI-MS analysis. Conjugates **10** and **15** displayed very high *in vitro* TSPO affinity and selectivity, and, hence, they may possess potential for targeted brain delivery. Due to the favorable features displayed by the conjugate **15**, it was further evaluated on glioma cell lines, expressing high levels of TSPO, in the presence and in the absence of specific nucleoside transport (NT) inhibitors. In contrast to that observed for the free Ara-C, the presence of NT inhibitors did not reduce the cytotoxic activity of **15**. Moreover, conjugate **15**, as N^4-acyl derivative of Ara-C, should be resistant to inactivation by cytidine deaminase, and it may possess enhanced propensity to target brain tumor cells characterized by a reduced expression of NTs. In addition, this conjugate behaves as a clear P-gp modulator and thereby may be useful to reverse MDR. Transport studies across the MDCKII-MDR1 monolayer indicated that conjugate **15** should overcome the BBB by transcellular pathway. All these features may be useful for enhancing the clinical potential of the nucleoside drug Ara-C.

Keywords: Ara-C (cytarabine); TSPO conjugates; stability; cytotoxicity; BBB; transport studies; brain tumors

Introduction

Peripheral-type benzodiazepine receptors for which the name "translocator protein (TSPO)" has recently been proposed[1] are abundant in peripheral organs like kidney, liver, lung as well as in glial cells of the central nervous

* To whom correspondence should be addressed. Mailing address: Dipartimento Farmaco-Chimico, Università degli Studi di Bari, Via Orabona 4, 70125 Bari, Italy. Phone: (039) 080-5442764. Fax: (039) 0805442724. E-mail: trapani@farmchim.uniba.it.

[†] Università degli Studi di Bari.

[‡] Mount Sinai School of Medicine.

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system (CNS).[2] The pharmacological action of TSPOs is completely different from the central-type benzodiazepine receptors (CBRs), which are located in neurons in the CNS. TSPOs are located primarily on mitochondrial membranes[2] within cells and are composed of at least three subunits: the isoquinoline-binding protein, a voltage-dependent anion channel, and an adenine nucleotide carrier.[3] The trimeric complex is a component of the mitochondrial permeability transitional (MPT) pore, which plays an important role in the passage of various biogenic compounds between the cytoplasm and mitochondrial intermembrane.[4] There is growing experimental evidence suggesting that TSPOs are involved in the regulation of cholesterol transport into mitochondria and in steroidogenesis[5] as well as in various neurological diseases such as Alzheimer's disease, Huntington's disease, epilepsy and stroke-induced brain injury.[6,7] It has also been shown that TSPO expression selectively increases in many tumor types, such as brain, colon, breast, and ovary, which suggests a possible role of these receptors in the tumorigenesis.[3,8−11] Moreover, there is evidence indicating TSPO-specific ligands induce apoptosis and cell cycle arrest in cancer cells,[3,12−15] which is consistent with the association of TSPOs with other mitochondrial proteins (i.e., the anti- and proapoptotic members of the Bcl-2 family) of the MPT pore known to be involved in apoptosis. The increased expression of TSPOs in neoplastic cells opens up the possibility to evaluate TSPO ligands as diagnostic imaging agents in oncology and to use them as receptor-mediated drug carriers to selectively target anticancer drugs to tumors. To date, the known TSPO-selective ligands belong to different chemical classes such as benzodiazepines (Ro-54864),[16] isoquinolines (PK-11195),[17] 2-aryl-3-indoleacetamides (FGIN-1−27),[18] and *N*-phenoxyphenyl-*N*-isopropoxybenzyl-acetamides (DAA1097)[19] (Figure 1). However, it should be noted that none of these TSPO ligands contain hydrophilic groups or organic functions such as amino, hydroxy and carboxylic groups useful for further conjugation. Development of conjugable forms of TSPO ligands would allow their use as starting material for the formulation of anticancer drug delivery systems and as diagnostic imaging agents as well. In this context, we have recently synthesized the first examples of conjugable imidazopyridinacetamide TSPO ligands endowed with high affinity and selectivity for TSPO (Figure 1).[20−22]

It is known that malignant brain tumors are among the most challenging to treat, and the limited survival advantage attributed to chemotherapy is partially due to low CNS

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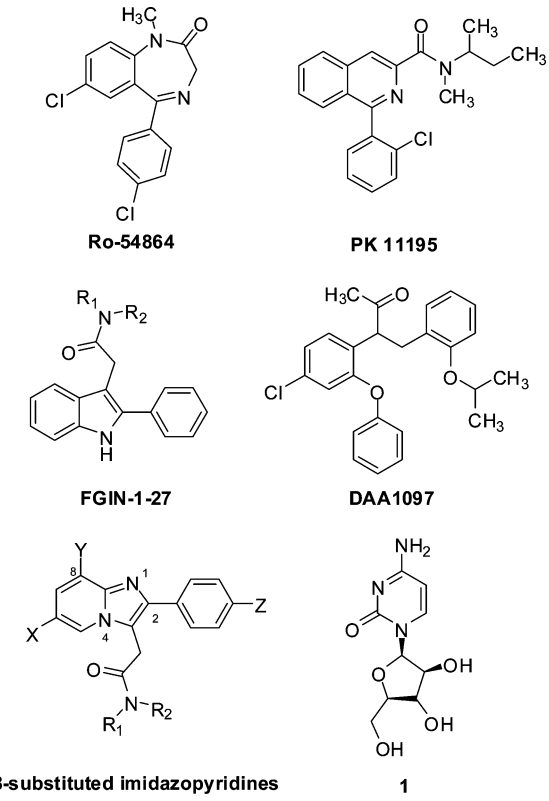

Figure 1. The chemical structures of the known TSPO-selective ligands and Ara-C (**1**).

penetration of antineoplastic agents across the blood−brain barrier (BBB). The structure of the normally tight junctions of the BBB in brain tumors may be compromised, leading to a disrupted and "leaky" barrier. However, other areas, particularly at the outer rim of the tumor, may have a more normal BBB where it is still a challenge to achieve effective drug delivery.[23] Moreover, cytotoxic anticancer drugs possess limited specificity and cause lethal damage to healthy cells. Based on these considerations and the observation that TSPOs can be overexpressed in brain tumors,[24−26] it is of interest to devise TSPO-based drug delivery systems to selectively target brain tumors and overcome the BBB.

The proof-of-concept of the potential of TSPO ligand−anticancer drug conjugate for targeted brain delivery was demonstrated by Guo et al. using a TSPO ligand−gemcitabine (GEM) conjugate in a preclinical brain tumor model.[24] The approximate 2-fold enhancement in brain tumor penetration compared with GEM alone was likely due to both the increased lipophilicity of the conjugate leading to enhanced transport across BBB[27] and, possibly, due to TSPO-mediated delivery to tumor cells. Based on these results, several efforts have been devoted to the identification and evaluation of new TSPO ligand−anticancer drug conjugates.[28,29]

Ara-C [cytarabine, cytosine arabinose, 1-(β-D-arabinofuranosyl)cytosine, Figure 1] is a pyrimidine nucleoside analogue employed for the treatment of various cancers including acute and chronic myeloblastic leukemia, colon, breast and ovary carcinoma.[30] AraC has also been part of combination chemotherapeutic regimens for various brain tumors including primary CNS lymphoma, and recurrent or refractory malignant gliomas.[31] In particular, promising results for the treatment of brain tumors were obtained using sustained delivery systems of cytarabine represented by a liposomal formulation, (i.e., Depocyte).[32]

Ara-C is rapidly converted to an inactive and more soluble metabolite 1-(β-D-arabinofuranosyl)uracil (Ara-U) by cy-

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tosine nucleoside deaminase that is widely distributed in tissues including hepatic and intestinal cells. Ara-C requires intracellular conversion to a triphosphate anabolite for biological activity that is initiated by the formation of a 5′-monophosphate moiety by deoxycytidine kinase. Many nucleoside analogues, including Ara-C, enter cells *via* specific nucleoside transporters (NTs). In this regard, it is also well-known that the clinical potential of Ara-C can be impaired by a deficient cellular drug uptake due to a reduced expression of NTs in cancer cells.[33] The complexities of Ara-C's membrane transport attributes, rapid clearance, and intracellular activation have promoted many efforts to modify its transport and metabolic properties to circumvent such deleterious attributes. Besides the use of high-dose protocols to ensure biological activity, many Ara-C prodrugs, such as polymeric-Ara-C conjugates, have been extensively examined.[34−37] In particular, N^4-amide- as well as 3′ and 5′ ester-Ara-C prodrugs have been synthesized with the aim to increase the Ara-C biological activity and/or to modify its pharmacokinetic properties.[35,36]

There have been no prior efforts to prepare TSPO ligand−Ara-C conjugates, which, in addition to an enhanced transport across BBB and a tumor targeting effect, might increase drug stability and prolong its efficacy. Therefore, the aim of this study was to synthesize and evaluate the cytotoxicity of imidazopyridinacetamide TSPO ligand−Ara-C (TSPO-Ara-C) conjugates in glioma cells as an approach that may serve to selectively deliver antineoplastic agent to brain tumors and improve the therapeutic potential.

Materials and Methods

Materials. The starting cytarabine hydrochloride **1**, di-*tert*-butyl dicarbonate (DBDC), 1-hydroxybenzotriazole (HOBZT), dicyclohexylcarbodiimide (DCC), triethylamine (TEA), 1,1′-carbonyldiimidazole (CDI), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide (EDC) and 4-(dimethylamino)pyridine (DMAP) were purchased from Sigma-Aldrich (Milan, Italy). The preparation of the imidazopyridine compounds **2a−c**, **8** and **11** has previously been reported.[20−22]

Apparatus. Melting points were determined in open capillary tubes with a Büchi apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer Spectrum one system spectrophotometer (KBr pellets for solid). ^{1}H NMR spectra were determined on a Varian VX Mercury instrument operating at 300 MHz. Chemical shifts are given in δ values. The mass spectra of all new compounds were obtained using an Agilent 1100 LC-MSD trap system VL instrument using methanol/ammonium formate 7 mM 9:1 (v/v). All compounds showed appropriate IR, ^{1}H NMR and mass spectra. Elemental analyses were performed on a Hewlett-Packard 185 C, H, N analyzer and agreed with theoretical values within ±0.40%. Thin layer chromatography (TLC) analyses were performed on silica gel plate 60 F254 (Merck). Silica gel 60 (Merck 70- 230 mesh) was used for column chromatography. All the following reactions were performed under a nitrogen atmosphere.

High-Performance Liquid Chromatography (HPLC) Analyses. HPLC analyses were performed with a Waters Associates model 600 pump equipped with a Waters 2996 photodiode array detector and Empower software or with a 990 variable wavelength UV detector. For kinetic studies on conjugates **3a−c**, **10**, and **15**, a reversed phase Symmetry C18 (25 cm ×3.9 mm; 5 μm particles) column in conjunction with a SecurityGuard Phenomenex precolumn was eluted with mixtures of methanol and deionized water 80/20 (v/v). The volume injected was 20 μL. The flow rate of 0.8 mL/min was maintained, and the column effluent was monitored continuously at 254 nm. Quantification of the compounds was carried out by measuring the peak areas in relation to those of standards chromatographed under the same conditions. Stability studies were carried out at controlled temperature at 37 °C (±0.2 °C) in a water bath.

For transport studies on compound **15** and diazepam, a reversed phase Phenomenex Synergi Hydro (250 × 4.6 mm; 4 μm particles) column in conjunction with a SecurityGuard Phenomenex precolumn was used with mixtures of methanol/25 mM ammonium acetate buffer pH 4.8 70/30 (v/v). A volume of 20 μL of the sample was injected, and the flow rate of 0.8 mL/min was maintained. The column effluent was monitored continuously at 254 nm. Quantification of the compounds was carried out as mentioned above. Analyses of Ara-C samples were performed similarly with a change in mobile phase containing 25 mM ammonium acetate buffer pH 4.8/methanol 95/5 (v/v), and the column effluent was monitored continuously at 275 nm.

Synthetic Procedures for TSPO ligand−Ara C Conjugates 3a−c, 10, 15. *General Procedure for the Preparation of Conjugates **3a−c**.* A solution of the appropriate imidazo[1,2-*a*]pyridine-3-acetic acid **2** (0.85 mmol) and CDI (1.0 mmol) in DMF (10 mL) was stirred at room temperature (rt) for 15 min. Then, cytarabine hydrochloride **1** (0.82 mmol) was added and stirring was prolonged overnight. Subsequently, the solvent was evapo-

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rated under reduced pressure and the residue was purified by silica gel column chromatography [chloroform/methanol 85/15 (v/v) as eluent] to give the corresponding conjugate **3**.

*2-(6,8-Dichloro-2-(4-chlorophenyl)imidazo[1,2-a]pyridin-3-yl)-N-(1-((2R,3S,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)acetamide (**3a**).* Yield: 25%. Mp: 176 °C dec. IR (KBr): 3233, 1722, 1654 cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.5−3.7 (m, 2H, 5′-CH_2O Ara-C), 3.7−4.1 (m, 3H, 2′, 3′,4′-CHO Ara-C), 4.40 (s, 2H, CH_2CO), 5.0−5.1 (m, 1H, 5′-OH Ara-C), 5.4−5.5 (m, 2H, 2′-OH + 3′-OH Ara-C), 6.05 (d, J = 3.8 Hz, 1H, 1′-CHO(N) Ara-C), 7.10 (d, J = 7.4 Hz, 1H, 5-CH Ara-C), 7.56 (d, J = 7.8 Hz, 2H, Ar), 7.7−7.8 (m, 3H, Ar), 8.07 (d, J = 7.4 Hz, 1H, 6-CHN Ara-C), 8.86 (d, J = 1.8 Hz, 1H, Ar), 11.2 (bs, 1H, NHCO). MS (ESI) *m/z*: 580 $[M + H]^+$. Anal. ($C_{24}H_{20}Cl_3N_5O_6$) C, H, N.

*2-(6,8-Dichloro-2-(phenyl)imidazo[1,2-a]pyridin-3-yl)-N-(1-((2R,3S,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)acetamide (**3b**).* Yield: 23%. Mp:140 °C dec. IR (KBr): 3388, 1721, 1648 cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.5−3.7 (m, 2H, 5′-CH_2O Ara-C), 3.7−4.1 (m, 3H, 2′, 3′,4′-CHO Ara-C), 4.41 (s, 2H, CH_2CO), 5.0−5.1 (m, 1H, 5′-OH Ara-C), 5.4−5.5 (m, 2H, 2′-OH + 3′-OH Ara-C), 6.05 (d, J = 3.8 Hz, 1H, 1′-CHO(N) Ara-C), 7.10 (d, J = 7.4 Hz, 1H, 5-CH Ara-C), 7.3−7.5 (m, 3H, Ar), 7.6−7.8 (m, 3H, Ar), 8.07 (d, J = 7.4 Hz, 1H, 6-CHN Ara-C), 8.86 (m, 1H, Ar), 11.3 (bs, 1H, NHCO). MS (ESI) *m/z*: 546 $[M + H]^+$. Anal. ($C_{24}H_{21}Cl_2N_5O_6$) C, H, N.

*2-(6-Chloro-2-(4-chlorophenyl)imidazo[1,2-a]pyridin-3-yl)-N-(1-((2R,3S,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)acetamide (**3c**).* Yield: 65%. Mp: 182−184 °C. IR (KBr): 3218, 1714, 1663 cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.5−3.7 (m, 2H, 5′-CH_2O Ara-C), 3.7−4.1 (m, 3H, 2′, 3′,4′-CHO Ara-C), 4.41 (s, 2H, CH_2CO), 5.0−5.1 (m, 1H, 5′-OH Ara-C), 5.4−5.5 (m, 2H, 2′-OH + 3′-OH Ara-C), 6.05 (d, J = 3.8 Hz, 1H, 1′-CHO(N) Ara-C), 6.95 (t, J = 7.4 Hz, 1H, Ar),7.10 (d, J = 7.4 Hz, 1H, 5-CH Ara-C), 7.4−7.6 (m, 3H, Ar), 7.75 (d, J = 8.5 Hz, 2H, Ar), 8.07 (d, J = 7.4 Hz, 1H, 6-CHN Ara-C), 8.45 (d, J = 6.9 Hz, 1H, Ar), 11.3 (bs, 1H, NHCO). MS (ESI) *m/z*: 546 $[M + H]^+$. Anal. ($C_{24}H_{21}Cl_2N_5O_6$) C, H, N.

*Preparation of (2R,3S,4R,5R)-2-(4-Amino-2-oxopyrimidin-1(2H)-yl)-4-(pivaloyloxy)-5-(pivaloyloxymethyl)tetrahydrofuran-3-yl3,3-Dimethylbutanoate(**4**)andtert-Butyl1-((2R,3S,4R,5R)-3,4-Bis(tert-butoxycarbonyloxy)-5-((tert-butoxycarbonyloxy)-methyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-ylcarbamate (**5**).* To a stirred solution of cytarabine hydrochlolride **1** (0.5 g, 1.8 mmol) in 20 mL of 1 N aqueous KOH, DBDC (3.9 g, 1.8 mmol) in 40 mL of dioxane was added dropwise. The reaction mixture was stirred at rt for 16 h and monitored by TLC ($CHCl_3$/CH_3OH 85:15). Then, the solvent was removed under reduced pressure and the residue was extracted with EtOAc (3 × 50 mL). The combined organic extracts were washed with water, dried over Na_2SO_4 and concentrated to dryness. The resulting residue and DBDC (3.9 g, 1.8 mmol) were dissolved in 40 mL of dioxane. To this solution with stirring and at rt was added 40 mL of 1 N aqueous KOH. The reaction progress was monitored by TLC. After 1 h the reaction was nearly complete to give compound **4** as the main product together with lower amounts of compound **5**. Then, the solvent was removed under reduced pressure and the residue purified by silica gel column chromatography (chloroform as eluent) to give compounds **4** and **5**.

4. Yield: 48%. Oil. IR (KBr): 3243, 1749, 1648 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.33 (s, 9H, CH_3), 1.44 (s, 18H, CH_3), 4.1−4.2 (m, 1H, 4′-CHO), 4.3−4.5 (m, 2H, 5′-CH_2O), 4.9−5.0 (m, 1H, 3′-CHO), 5.2−5.3 (m, 1H, 2′-CHO), 5.78 (d, J = 7.4 Hz, 1H, 5-CH), 6.2−6.4 (m, 1H, 1′-CHO(N)), 7.46 (d, J = 7.4 Hz, 1H, 6-CHN). MS (ESI) *m/z*: 542 $[M - H]^-$.

5. Yield: 15%. Oil. IR (KBr): 1747, 1681 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.33 (s, 9H, CH_3), 1.44 (s, 18H, CH_3), 4.1−4.2 (m, 1H, 4′-CHO), 4.3−4.5 (m, 2H, 5′-CH_2O), 4.9−5.0 (m, 1H, 3′-CHO), 5.2−5.3 (m, 1H, 2′-CHO), 5.78 (d, J = 7.4 Hz, 1H, 5-CH), 6.2−6.4 (m, 1H, 1′-CHO(N)), 7.46 (d, J = 7.4 Hz, 1H, 6-CHN).

*Preparation of ((2R,3R,4S,5R)-5-(4-(tert-Butoxycarbonylamino)-2-oxopyrimidin-1(2H)-yl)-3,4-bis(tert-butoxycarbonyloxy)-tetrahydrofuran-2-yl)methyl 2-(6,8-Dichloro-2-(4-chlorophenyl)imidazo[1,2-a]pyridin-3-yl)acetate (**6**) and (2R,3S,4R,5R)-2-(4-(2-(6,8-Dichloro-2-(4-chlorophenyl)imidazo[1,2-a]pyridin-3-yl)acetamido)-2-oxopyrimidin-1(2H)-yl)-4-(pivaloyloxy)-5-(pivaloyloxymethyl)tetrahydrofuran-3-yl 3,3-Dimethylbutanoate (**7**).* To a stirred suspension of the 2-(4-chlorophenyl)-6,8-dichloroimidazo [1,2-*a*]pyridine-3-acetic acid **2a** (212 mg, 0.6 mmol) in anhydrous CH_2Cl_2 (20 mL) were added HOBZT (90 mg, 0.66 mmol) and DCC (136 mg, 0.66 mmol) at rt. After 30 min, compound **4** (296 mg, 0.55 mmol) was added, and stirring was prolonged for 72 h. Then, ethyl ether was added and the resulting dicyclohexylurea precipitate removed by filtration. The filtrate was concentrated, and the residue was purified by silica gel column chromatography [chloroform/methanol 85/15 (v/v) as eluent)] to give the protected conjugates **6** and **7**.

6. Yield: 42%. Mp: 152 °C dec. IR (KBr): 1747, 1665 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.31 (s, 9H, CH_3), 1.50 (s, 9H, CH_3), 1.51 (s, 9H, CH_3), 4.11 (s, 2H, CH_2CO), 4.2−4.7 (m, 4H, 5′-CH_2O and 3′,4′-CHO Ara-C), 5.0−5.1 (m, 1H, 2′-CHO Ara-C), 6.35 (d, J = 3.0 Hz, 1H, 1′-CHO(N) Ara-C), 7.15 (d, J = 7.7 Hz, 1H, 5-CH Ara-C), 7.31 (d, J = 1.6 Hz, 1H, Ar), 7.44 (d, J = 8.5 Hz, 2H, Ar), 7.3−7.5 (m, 3H, 6-CH Ara-C and Ar), 8.13 (d, J = 1.8 Hz, 1H, Ar). MS (ESI) *m/z*: 880 $[M - H]^-$.

7. Yield: 49%. Mp: 148 °C dec. IR (KBr): 3442, 1736, 1652 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.42 (s, 9H, CH_3), 1.44 (s, 9H, CH_3), 1.48 (s, 9H, CH_3), 3.8−4.0 (m, 2H, CH_2CO), 4.2−4.6 (m, 4H, 5′-CH_2O and 3′,4′-CHO Ara-C), 5.0−5.1 (m, 1H, 2′-CHO Ara-C), 6.13 (d, J = 4.9 Hz, 1H, 1′-CHO(N) Ara-C), 6.97 (d, J = 7.7 Hz, 1H, 5-CH Ara-C), 7.29 (d, J = 1.6 Hz, 1H, Ar), 7.39 (d, J = 8.5 Hz, 2H, Ar), 7.58 (d, J = 8.5 Hz, 2H, Ar), 7.75 (d, J = 7.7 Hz, 1H, 6-CHN Ara-C), 8.18 (d, J = 1.6 Hz, 1H, Ar). MS (ESI) *m/z*: 880 $[M - H]^-$.

Deprotection Reaction of Compound ***7.*** To a stirred solution of **7** (100 mg) in CH_2Cl_2 (10 mL) at rt was added TFA (5 mL), and the reaction progress was monitored by TLC ($CHCl_3$/CH_3OH 85:15 v/v as eluent) for 2 h. Then, the stirring was prolonged overnight and, subsequently, the solvent was removed under reduced pressure to give the crude conjugate **3a** in almost quantitative yield.

Procedure for the Preparation of Conjugate ***10.*** A solution of the 2-(6,8-dichloro-2-(4-hydroxyphenyl)imidazo[1,2-*a*]pyridin-3-yl)-*N*,*N*-dipropylacetamide **8** (0.24 mmol) and 4-nitrophenyl chloroformate (0.48 mmol) in anhydrous THF (10 mL) was stirred at rt for 6 h in the presence of TEA. Then, to the obtained intermediate **9**, a solution of cytarabine hydrochloride **1** (0.24 mmol) in 2 mL of anhydrous DMF was added and stirring was prolonged overnight. Afterward, solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography [chloroform/methanol 95/5 (v/v) as eluent] to give the corresponding conjugate **10**.

*4-(6,8-Dichloro-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-2-yl)phenyl 1-((2R,3S,4S,5R)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-ylcarbamate (**10**).* Yield: 23%. Mp: 156 °C dec. IR (KBr): 3327, 1704, 1627 cm^{-1}. ^1H NMR (DMSO-d_6) δ: 0.7−0.9 (m, 6H, CH_3), 1.4−1.7 (m, 4H, CH_2), 3.1−3.4 (m, 4H, $-CH_2NCO$), 3.1−3.4 (m, 2H, 5′-CH_2O Ara-C), 3.8−4.1 (m, 3H, 2′, 3′,4′-CHO Ara-C), 4.28 (s, 2H, $-CH_2CON$), 6.12 (d, J = 3.57 Hz, 1H, 1′-CHO(N) Ara-C), 6.82 (d, J = 7.4 Hz, 1H, 5-CH Ara-C), 7.52 (d, J = 7.4 Hz, 2H, Ar), 7.6−7.7 (m, 3H, Ar), 8.05 (d, J = 7.4 Hz, 1H, 6-CHN Ara-C), 8.60 (d, J = 1.6 Hz, 1H, Ar). MS (ESI) *m*/*z*: 711 $[M + Na]^+$. Anal. ($C_{31}H_{34}Cl_2N_6O_8$) C, H, N.

Procedure for the Preparation of Conjugate ***15.*** A mixture of compound **13** (0.36 mmol), 2-mercaptothiazoline (0.43 mmol), EDC·HCl (0.39 mmol), and DMAP (0.43 mmol) in anhydrous CH_2Cl_2 (20 mL) was stirred for 4 h at rt. The mixture was concentrated *in vacuo*, and then the residue was purified by silica gel column chromatography [chloroform/acetone 8/2 (v/v) as eluent] to give the pure compound **14**. Then, a mixture of **14** (0.07 mmol) and **1** (0.14 mmol) in anhydrous pyridine was stirred at 50 °C overnight. After that, the reaction mixture was concentrated *in vacuo* and the residue purified by silica gel column chromatography [chloroform/methanol 85/15 (v/v) as eluent] to give the corresponding conjugate **15**. Compound **13**, in turn, was synthesized following a two step procedure. First, the 2-(8-amino-2-(4-chlorophenyl)imidazo[1,2-*a*]pyridin-3-yl)-*N*,*N*-dipropylacetamide **11** (0.13 mmol) was dissolved in anhydrous THF at rt in the presence of K_2CO_3 (0.65 mmol) and methyl adipoyl chloride (0.26 mmol). The resulting solution was stirred at rt for 2 h. After that period, the solvent was removed under reduced pressure and the residue dissolved in ethyl acetate and extracted with brine. The organic phase was dried on anhydrous Na_2SO_4 and evaporated to dryness, giving compound **12**. Second, to a solution of the methyl ester **12** in dioxane (10 mL) was added NaOH 0.1 N (10 mL). The mixture was stirred at 50 °C for 1 h, and then, the solvent was evaporated under reduced pressure. The residue was taken up with water, and the cooled aqueous solution was acidified with dilute HCl until pH 4 was reached. The resulting precipitate was the pure compound **13**, which was isolated by filtration.

*Methyl 6-(2-(4-Chlorophenyl)-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-8-ylamino)-6-oxohexanoate (**12**).* Yield: 67%. Mp: 136 °C dec. IR (KBr): 3393, 1736, 1694, 1639 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.75 (t, J = 7.4 Hz, 3H, CH_3), 0.84 (t, J = 7.4 Hz, 3H, CH_3), 1.4−1.6 (m, 4H, CH_2), 1.6−2.0 (m, 4H, CH_2), 2.37 (t, J = 7.15 Hz, 2H, CH_2CON), 2.53 (t, J = 7.15 Hz, 2H, CH_2COO), 3.11 (t, J = 7.4 Hz, 2H, CH_2NCO), 3.28 (t, J = 7.4 Hz, 2H, CH_2NCO), 3.67 (s, 3H, CH_3O), 4.05 (s, 2H, CH_2CON), 6.83 (t, J = 7.1 Hz, 1H, Ar), 7.45 (d, J = 8.5 Hz, 2H, Ar), 7.60 (d, J = 8.5 Hz, 2H, Ar), 7.90 (d, J = 7.4 Hz, 1H, Ar), 8.19 (d, J = 7.4 Hz, 1H, Ar), 8.71 (b, 1H, NH). MS (ESI) *m*/*z*: 565 $[M + K]^+$. Anal. ($C_{28}H_{35}ClN_4O_4$) C, H, N.

*6-(2-(4-Chlorophenyl)-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-8-ylamino)-6-oxohexanoic Acid (**13**).* Yield: 88%. Mp: 125 °C dec. IR (KBr): 1659, 1638, 1551 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.75 (t, J = 7.4 Hz, 3H, CH_3), 0.84 (t, J = 7.4 Hz, 3H, CH_3), 1.4−1.6 (m, 4H, CH_2), 1.6−1.8 (m, 4H, CH_2), 2.35 (t, J = 6.8 Hz, 2H, CH_2CON), 2.56 (t, J = 6.8 Hz, 2H, CH_2COO), 3.08 (t, J = 7.7 Hz, 2H, CH_2NCO), 3.27 (t, J = 7.7 Hz, 2H, CH_2NCO), 3.98 (s, 2H, CH_2CON), 6.89 (t, J = 7.1 Hz, 1H, Ar), 7.43 (d, J = 8.5 Hz, 2H, Ar), 7.46 (d, J = 8.5 Hz, 2H, Ar), 7.91 (d, J = 6.9 Hz, 1H, Ar), 8.30 (d, J = 6.9 Hz, 1H, Ar), 9.66 (b, 1H, NH). MS (ESI) *m*/*z*: 511 $[M - H]^-$. Anal. ($C_{27}H_{33}ClN_4O_4$) C, H, N.

*N-(2-(4-Chlorophenyl)-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-8-yl)-6-oxo-6-(2-thioxothiazolidin-3-yl)hexanamide (**14**).* Yield: 72%. Mp: 165 °C dec. IR (KBr): 1698, 1636 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.74 (t, J = 7.4 Hz, 3H, CH_3), 0.84 (t, J = 7.4 Hz, 3H, CH_3), 1.4−1.6 (m, 4H, CH_2), 1.7−2.0 (m, 4H, CH_2), 2.5−2.6 (m, 2H, CH_2S), 3.11 (t, J = 7.7 Hz, 2H, CH_2NCO), 3.2−3.4 (m, 2H, CH_2NCO), 3.2−3.4 (m, 4H, CH_2CON), 4.06 (s, 2H, CH_2CON), 4.57 (t, J = 7.4 Hz, 2H, CH_2N), 6.82 (t, J = 7.1 Hz, 1H, Ar), 7.27 (d, J = 8.5 Hz, 2H, Ar), 7.45 (d, J = 8.5 Hz, 2H, Ar), 7.90 (d, J = 7.4 Hz, 1H, Ar), 8.17 (d, J = 7.4 Hz, 1H, Ar), 8.56 (b, 1H, NH). MS (ESI) *m*/*z*: 636 $[M + Na]^+$. Anal. ($C_{30}H_{36}ClN_5O_3S_2$) C, H, N.

*N1-(2-(4-Chlorophenyl)-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-8-yl)-N6-(1-((2R,3S,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)adipamide (**15**).* Yield: 66%. Mp: 125 °C dec. IR (KBr): 1643, 1553 cm^{-1}. ^1H NMR (DMSO-d_6) δ:0.7−0.9 (m, 6H, CH_3), 1.4−1.6 (m, 4H, CH_2), 2.4−2.6 (m, 4H, CH_2), 3.2−3.4 (m, 4H, CH_2NCO), 3.2−3.4 (m, 4H, CH_2CON), 3.5−3.7 (m, 2H, CH_2O Ara-C), 3.7−4.2 (m, 3H, CHO Ara-C), 4.19 (s, 2H, CH_2CON), 6.02 (d, J = 3.85 Hz, 1H, 1′-CHO(N) Ara-C), 6.87 (t, J = 6.9 Hz, 1H, Ar), 7.19 (d, J = 7.4 Hz, 1H, 5-CH Ara-C), 7.51 (d, J = 8.5 Hz, 2H, Ar), 7.67 (d, J = 8.5 Hz, 2H, Ar), 7.8−8.0 (m, 2H, Ar), 8.03 (d, J = 7.4 Hz, 1H, 6-CH Ara-C), 9.77 (s, 1H, NH), 10.8 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 11.6, 11.9, 21.1, 22.4, 24.9, 25.3, 29.7, 36.5, 36.9, 40.1, 47.7, 49.3, 49.6, 61.7, 75.3,

76.8, 86.4, 87.7, 94.9, 111.9, 112.8, 118.3, 120.2, 127.7, 129.2, 130.3, 132.9, 133.9, 138.9, 141.0, 147.4, 155.2, 162.8, 168.1, 173.3, 174.4. MS (ESI) *m/z*: 760 $[M + Na]^+$. Anal. ($C_{36}H_{44}ClN_7O_8$) C, H, N.

Stability Studies. *Chemical Hydrolysis.* The hydrolysis of the Ara-C conjugates **3**, **10** and **15** was studied at pH 7.4 in 0.05 M phosphate buffer at 37 ± 0.2 °C in a water bath. The reaction was carried out by adding 100 μL of a stock solution of the conjugates (5 mg/mL in methanol) to 5 mL of the buffer solution preheated at 37 °C. The final concentration of the compounds was about 1×10^{-5} M. The resulting solutions were vortexed and maintained in a water bath at constant temperature of 37 ± 0.2 °C. Aliquots of 400 μL were removed at appropriate intervals and either immediately analyzed or frozen at −20 °C until analyzed by HPLC. Each sample was filtered through a 0.2 μm membrane filter (cellulose acetate) and then analyzed by HPLC. Pseudo-first-order rate constants for the hydrolysis of **3**, **10** and **15** were determined from the slopes of linear plots of the logarithms of residual starting material against time.

Stability in Physiological Medium. The stability in physiological medium of the Ara-C conjugates **3**, **10** and **15** was studied at 37 °C in 0.05 M phosphate buffer and 0.14 M NaCl at pH 7.4, containing 50% v/v of human serum. The reaction was carried out by adding 100 μL of the stock solution of compound in methanol (5 mg/5 mL) to 1.6 mL of preheated serum solution, and the mixture was maintained in water bath at 37 ± 0.2 °C. Aliquots of 100 μL were withdrawn at appropriate intervals and added to 500 μL of cold acetonitrile in order to deproteinize the serum. After mixing and centrifugation for 10 min at 2000 rpm, 20 μL of the clear supernatant was analyzed by HPLC. Pseudo-first-order rate constants for the degradation of compounds **3**, **10** and **15** were determined from the slopes of linear plots of the logarithms of remaining Ara-C conjugate against time. Degradation products of Ara-C conjugates **3**, **10** and **15** in serum, were characterized by direct infusion of an aliquot of deproteinized supernatant diluted 1:10 with blank by ESI (both positive and negative mode).

Biological Methods. Adult male or female Sprague−Dawley CD rats (Charles River, Como, Italy) with body masses of 200−250 g at the beginning the experiments were maintained under an artificial 12 h light/dark cycle (light on 08.00 to 20.00 h) at a constant temperature of 23 ± 2 °C and 65% humidity. Food and water were freely available, and the animals were acclimatized for >7 days before use. Experiments were performed between 08.00 and 14.00 h. Animal care and handling througouth the experimental procedure were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocol was approved by the Animal Ethical Committee of the University of Cagliari (Italy).

In Vitro Receptor Binding Assays. After sacrifice the brain was rapidly removed, the cerebral cortex was dissected and tissues were stored at −80 °C until assay.

[^{3}H]Flunitrazepam Binding. The tissues were thawed and homogenized with a Polytron PT 10 in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and centrifuged twice at 20000*g* for 10 min. The pellet was reconstituted in 50 volumes of Tris-HCl buffer and was used for the binding assay. Aliquots of 400 μL of tissue homogenate (0.4−0.5 mg of protein) were incubated in presence of [^{3}H]flunitrazepam at a final concentration of 0.5 nM, in a total incubation volume of 1000 μL. The drugs were added in 100 μL aliquots. After a 60 min incubation at 0 °C, the assay was determined by rapid filtration through glass-fiber filter strips (Whatman GF/B). The filters were rinsed with 2 to 4 mL portions of ice-cold Tris-HCl buffer as described above. Radioactivity bound to the filters was quantitated by liquid scintillation spectrometry. Nonspecific binding was determined as binding in the presence of 5 μM diazepam and represented about 10% of total binding.

[^{3}H]PK 11195 Binding. The tissues were thawed and homogenized in 50 volumes of Dulbecco's phosphate buffered saline (PBS) pH 7.4 at 4 °C with a Polytron PT 10 (setting 5, for 20s). The homogenate was centrifuged at 40000*g* for 30 min, and the pellet was resuspended in 50 volumes of PBS and recentrifugated. The new pellet was resuspended in 20 volumes of PBS and used for the assay. [^{3}H]PK 11195 binding was determined in a final volume of 1000 μL of tissue homogenate (0.15−0.20 mg protein), 100 μL of [^{3}H]PK 11195 (sp act. 85.5 Ci/mmol, New England Nuclear) at final assay concentration of 1 nM, 5 μL of drug solution or solvent and 795 μL of PBS buffer (pH 7.4 at 25 °C). Incubations (25 °C) were initiated by addition of membranes and were terminated 90 min later by rapid filtration through glass-fiber filter strips (Wathaman GF/B), which were rinsed with five 4 mL portions of ice-cold PBS buffer using a cell harvester filtration manifold (Brandel). Filter bound radioactivity was quantified by liquid scintillation spectrometry. Nonspecific binding was defined as binding in the presence of 10 μM unlabeled PK 11195 (Sigma).

Cytotoxicity Assays of Conjugates *3a−c*, *10* and *15* against Human Glioma Cells Expressing High Levels of TSPO. SF126, SF188, RG2, and C6 glioma cells expressing high levels of TSPO were cultured in 96-well plates (1000 to 3000 cells/well) for 24 h prior to drug treatment. The wells containing culture medium were utilized as control. After the growth period, the cells were treated with either Ara-C or one of the conjugates **3a−c**, **10** and **15** at different concentrations for 72 h. Cell lines were also treated with vehicle (DMSO) only as controls. After the drug incubation period cytotoxicity assays were performed with CCK-8 (Alexis Biochemicals) according to the instructions from the manufacturer, followed by colorimetric measurements obtained with a microplate reader.

Cytotoxicity Assays of Conjugate *15* and Ara-C against C6 Glioma Cells in the Presence of Nucleoside Transport Inhibitors. C6 glioma cells were cultured in Ham's/F12 nutrient supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL strepto-

mycin. Cells were seeded in 96-well plates at a density of ~10000 cells/well, and, after 1 day of incubation at 37 °C in a humidified atmosphere with 5% CO_2, the culture medium was replaced with the same volume of fresh complete medium or with medium containing different concentrations of the tested compounds in the presence or not of the nucleoside transport inhibitor. The nucleoside transport inhibitors *S*-(4-nitrobenzyl)-6-thioinosine (NBTI, Sigma-Aldrich) and dipyridamole (Sigma-Aldrich) were used at subtoxic levels (100 μM and 30 μM respectively), while Ara-C and compound **15** were added at serial dilutions in the appropriate range concentrations. Untreated cells were used as positive control, and cells incubated with a 2% (w/v) SDS solution were used as negative control. In each well, the final volume was 200 μL. The cells were then allowed to proliferate for 72 h at 37 °C in a humidified CO_2-controlled atmosphere. Cytotoxicity values (IC_{50}) for **15** were determined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[38] At the end of the incubation period, 10 μL of a 0.5% (w/v) MTT/PBS solution was added to each well and the incubation was prolonged for a further 4 h. Then, medium was removed and replaced with 150 μL of a DMSO/ethanol (1:1) solution per well. The absorbance of the individual well was measured by microplate reader (Wallac Victor^3, 1420 Multilabel Counter, Perkin-Elmer). Each drug concentration was tested in triplicate, and the experiments were repeated three times.

***In Vitro* Assays To Predict Compound 15 P-Glycoprotein Interaction.** *Monolayer Efflux Studies.* [39] Apical to basolateral (P_{app}, AP) and basolateral to apical (P_{app}, BL) permeability of conjugate **15** were measured using human colonic carcinoma cells (Caco-2) monolayer grown on a MultiScreen assay system (Millipore). After 21 days of cell growth, the medium was removed from filter wells and from the receiver plate. The formation of confluent Caco-2 monolayer with tight junctions was confirmed by TEER values. The filter wells were filled with 75 μL of fresh Hanks' balanced salt solution (HBSS) buffer and the receiver plate with 250 μL per well of the same buffer. This procedure was repeated twice, and the plates were incubated at 37 °C for 30 min. Compound **15** was dissolved in HBSS pH 7.4 and sterile filtered. For AP-to-BL or BL-to-AP flux studies, the drug solution was added in the AP chamber (75 μL) or in the BL chamber (250 μL), respectively. The plates were incubated at 37 °C for 120 min. After incubation time, samples were removed from the apical and basolateral side of the monolayer and then analyzed by UV spectroscopy. The apparent permeability, in units of nm/s, was calculated using eq 1,

$$P_{app} = \left(\frac{V_A}{\text{area} \times \text{time}}\right) \times \left(\frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{initial}}}\right) \quad (1)$$

where V_A is the volume in the acceptor well, area is the surface area of the membrane, time is the total transport time, $[\text{drug}]_{\text{acceptor}}$ is the concentration of the drug measured by UV spectroscopy and $[\text{drug}]_{\text{initial}}$ is the initial drug concentration in the AP or BL chamber.

Calcein AM Inhibition Assay. This experiment was carried out as described by Feng et al.[39] with minor modifications. Madin-Darby canine kidney (i.e., MDCK) cells, retrovirally transfected with the human MDR1 cDNA (MDCKII-MDR1) (kindly provided by Prof. P. Borst, NKI-AVL Institute, Amsterdam, The Netherlands), were cultured in DMEM high glucose supplemented with 10% heat inactivated FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded at a density of ~50,000 cells per well into black 96-well plates and allowed to become confluent overnight. Compound **15** was added to monolayers in 100 μL of culture medium and incubated at 37 °C for 30 min. Calcein AM was added in 100 μL of PBS to yield a final concentration of 2.5 μM, and the plate was incubated for a further 30 min. Cells were then washed three times with ice-cold PBS. PBS was added to each well, and the plate was read with a Victor^3 fluorometer (Wallac Victor^3, 1420 Multilabel Counter, Perkin-Elmer) at excitation and emission wavelengths of 485 and 535 nm, respectively. In these experimental conditions, calcein cell accumulation, in the absence and in the presence of the tested compound **15**, was evaluated and fluorescence basal level was estimated by untreated cells. In treated wells the increase of fluorescence with respect to basal level was measured. IC_{50} values were determined by fitting the fluorescence increase percentage versus log[dose]. Elacridar, a known inhibitor of P-gp, was used as control.[40]

P-gp ATPase Activity Assay. The experiment was performed as reported in the technical sheet of ATPlite 1step kit for luminescence ATP detection. MDCKII-MDR1 cells were seeded into 96-well microplates in 100 μL of complete medium at a density of 2×10^4 cells/well. The plate was incubated overnight in a humidified atmosphere of 5% CO_2 at 37 °C. Then, the medium was removed and 100 μL of the complete medium in the presence or absence of different concentrations of conjugate **15** ranging from 1 to 100 μM was added. The plate was incubated for 2 h, and then 50 μL of mammalian cell lysis solution was added to all wells and the plate stirred for 5 min. In all wells, 50 μL of substrate solution was added, the plate stirred for 5 min was dark adapted for 10 min and in the end the luminescence was measured by using a Victor^3 luminometer (Wallac Victor^3, 1420 Multilabel Counter, Perkin-Elmer).

*Transport Studies on **15** through MDCKII-MDR1 Monolayer.* Transport studies on **15** were carried out using MDCKII-MDR1 cells monolayer grown on a 12-well Tran-

(38) Hansen, M.; Nielsen, S.; Berg, K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Immunol. Methods* **1989**, *119*, 203–210.

(39) Feng, B.; Mills, J. B.; Davidson, R. E.; Mireles, R. J.; Janiszewski, J. S.; Troutman, M. D.; de Morais, S. M. In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system. *Drug Metab. Dispos.* **2008**, *36*, 268–275.

(40) Hyafil, F.; Vergely, C.; Du Vignaud, P.; Grand-Perret, T. In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. *Cancer Res.* **1993**, *53*, 4595–4602.

swell insert (diameter 12 mm) as previously described.[41] All experiments were done at 37 °C. Medium was aspirated from both the apical (AP) and basolateral (BL) chambers of each insert, and cell monolayers were washed three times (10 min per wash) with DPBS pH 7.4. The formation of confluent MDCKII-MDR1 monolayer with tight junctions was confirmed by microscopy and TEER values. Diazepam (75 μM) and fluorescein isothiocyanate dextran (FD4, Sigma) (200 μg/mL) were used as markers for the transcellular and paracellular pathways, respectively. The assay medium was as follows: 0.4 mM K_2HPO_4, 25 mM $NaHCO_3$, 3 mM KCl, 122 mM NaCl, 10 mM glucose. The pH was 7.4, and the osmolarity was 300 mOsm as determined by a freeze point based osmometer. Except for FD4, which was solubilized directly in the assay medium, the other compounds were first dissolved in DMSO and then diluted with the assay medium to a final concentration of 75 μM. Next, the tested solutions were added to the donor side (0.5 mL for the AP chamber and 1.5 mL for the BL chamber) and fresh assay medium was placed in the receiver compartment. The percentage of DMSO never exceeded 1% (v/v) in the samples. During a period of 180 min, aliquots of 300 μL were withdrawn from the receiver side at scheduled intervals, and replaced with fresh assay medium to maintain sink conditions. Samples were stored at −20 °C until further analysis. The apparent permeability coefficient (P_{app}) was calculated according to eq 2,

$$P_{app} = \frac{V_A \times dC}{\text{area} \times [\text{drug}]_{initial} \times dt} \quad (2)$$

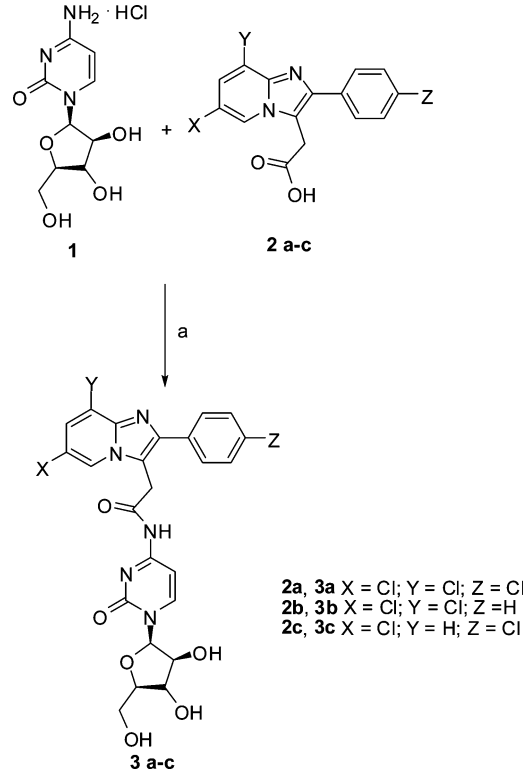
where $V_A \times dc/dt$ is the linear appearance rate of mass in the receiver solution, area is the filter/cell surface area and $[\text{drug}]_{initial}$ is the initial tested compound concentration in the AP or BL chamber.

Compound **15**, diazepam and Ara-C samples were analyzed by HPLC as described above. The FD4 samples were analyzed with a Victor^3 fluorometer (Wallac Victor^3, 1420 Multilabel Counter, Perkin-Elmer) at excitation and emission wavelengths of 485 and 535 nm, respectively. Each compound was tested in triplicate, and the experiments were repeated three times. Following the described protocol, transport studies on **15** were also carried out in the presence of known P-gp inhibitors such as verapamil (50 and 100 μM) and elacridar (2 μM).

Statistical Analysis. The statistical analysis was accomplished using one-way analysis of variance (ANOVA) followed by the Tukey post hoc tests (GraphPad Prism version 4 for Windows, GraphPad Software, San Diego, CA). Differences were considered statistically significant at $p < 0.05$.

(41) Denora, N.; Laquintana, V.; Lopedota, A.; Serra, M.; Dazzi, L.; Biggio, G.; Pal, D.; Mitra, A. K.; Latrofa, A.; Trapani, G.; Liso, G. Novel L-dopa and dopamine prodrugs containing a 2-phenylimidazopyridine moiety. *Pharm. Res.* **2007**, *24*, 1309–1324.

Scheme 1[a]



[a] (a) CDI, DMF.

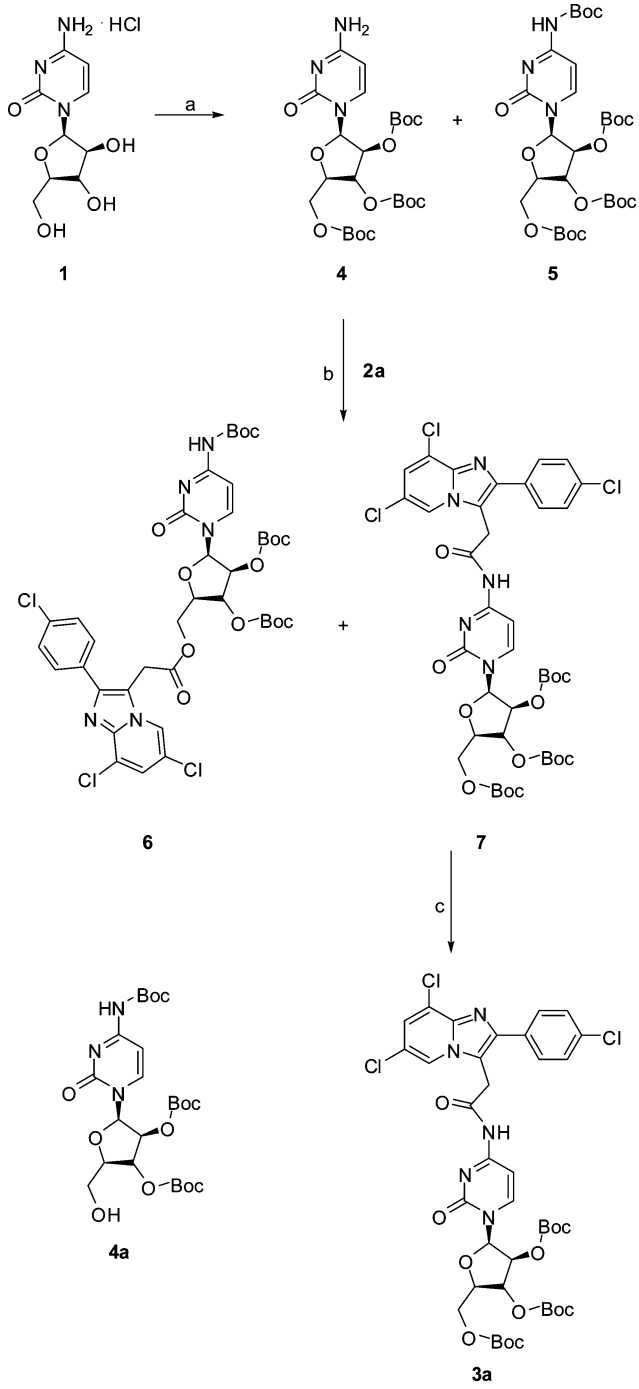
Results

Synthetic Procedures. As shown in Scheme 1, the new TSPO ligand−Ara-C conjugates **3a−c** were prepared by condensation of the corresponding imidazopyridine-acetic acids **2a−c** with Ara-C·HCl **1**. The condensation was successfully achieved by using CDI as dehydrating agent in anhydrous THF (Scheme 1). The Ara-C conjugates **3a−c** were obtained in moderate to good yields and were fully characterized by IR, ^{1}H NMR, mass spectra, and elemental analyses. An interesting feature of the ^{1}H NMR spectra of the Ara-C-imidazoacetamides **3a−c** concerns the signals due to the C(5)- and C(6)-protons in conjugates **3a−c**. These protons resonate at lower fields than the corresponding protons of Ara-C ($\Delta\delta$ 1.04 ppm and 0.15 ppm, respectively). This may be attributed to the deshielding effect of the N^4-carbonyl group in **3a−c**.

To confirm the structural assignments done, we decided to prepare **3a** by an alternative and unambiguous synthesis which involves the use of Boc protected Ara-C derivatives as shown in Scheme 2. Treatment of **1** with DTBDC in dioxane and successive alkalinization with KOH 1 N gave a mixture of tri- and tetra-Boc protected Ara-C derivatives (i.e., compounds **4** and **5**). Actually, when DBDC was added to a solution of **1** in dioxane−aqueous KOH, the product profile changed over the time.[42] To avoid the complete

(42) Guo, Z.; Gallo, J. M. Selective protection of 2′,2′-difluorodeoxycytidine (Gemcitabine). *J. Org. Chem.* **1999**, *64*, 8319–8322.

Scheme 2[a]



[a] (a) Dioxane−1 N aqueous KOH, DBDC; (b) DCC, HO-BZT; (c) TFA.

formation of the unreactive tetra-Boc protected compound **5**, it is necessary to add the DBDC stepwise. Compounds **4** and **5** were obtained prolonging the reaction time for 16 h at room temperature, and the mixture was separated by column chromatography on silica gel. The condensation of **2a** with **4** to give the desired compound **7** in 49% yield was successfully achieved by using DCC and HO-BZT as a dehydrating agent in CH_2Cl_2 at room temperature. Furthermore, formation of the isomer compound **6** was also found to occur in 42% yield. Likewise compound **4** undergoes a transacylation reaction to give the intermediate primary alcohol **4a**, which, in turn, can be esterified with **2a**, leading to compound **6**. Cleavage of the Boc groups in compound **7** was accomplished with TFA to give in almost quantitative yield **3a** identical in all the physicochemical features to that prepared according to Scheme 1.

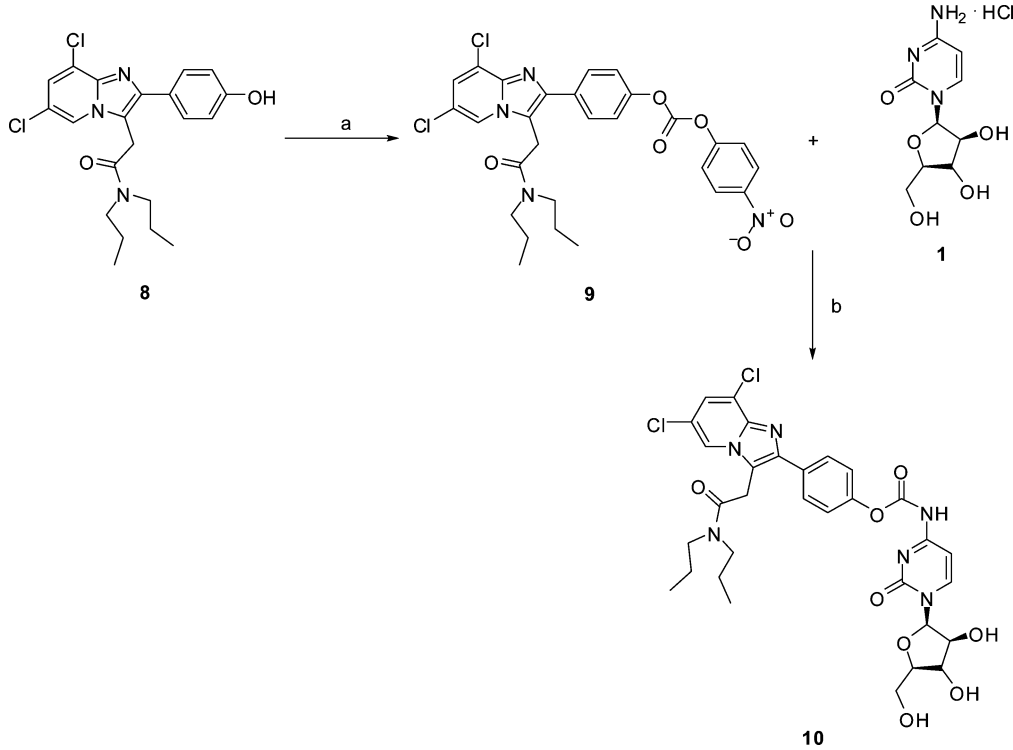
Preparation of conjugate **10** was accomplished according to the synthetic sequences showed in Scheme 3. Treatment of the TSPO ligand **8**[22] with 4-nitrophenyl chloroformate in anhydrous THF and in the presence of TEA gave the corresponding carbonate **9**, which in turn was condensed with **1**, yielding the carbamate conjugate **10** in moderate yield.

In Scheme 4 is shown the preparation of conjugate **15** involving the reaction of the TSPO ligand **11**[20] with methyl adipoyl chloride in the presence of K_2CO_3 to give the ester **12**, which was hydrolyzed to the corresponding acid **13**. To obtain the selective amidification of the N^4-amino group of Ara-C **1**, compound **13** was first activated with 2-mercaptothiazoline to yield the active thiazolidine thione **14**[35] and then condensed with **1** in anhydrous pyridine at 50 °C to produce the desired conjugate **15**.

Hydrolysis in Buffer and Physiological Media. The hydrolysis of the derivatives **3a**−**c**, **10** and **15** was determined in 0.05 M phosphate buffer at pH 7.4 as well as in 50% (v/v) dilute human serum solution at 37 °C. All the experiments were done in duplicate and half-lives were measured by the disappearance of the Ara-C conjugate. All the amide derivatives **3a**−**c** were relatively stable in 0.05 M phosphate buffer at pH 7.4 and their half-lives exceeding 13 h (Table 1). Conversely, compounds **3a**−**c** were found to be susceptible to enzyme-catalyzed hydrolysis in serum with half-lives in the range of 2−4.6 h (Table 1).

As for conjugates **10** and **15** a marked difference in their stability behavior was noted. In fact, compound **15** resulted to be very stable in both buffer and physiological medium with half-lives of 144 and 18 h, respectively, while conjugate **10** was found unstable in both conditions. It should be noted that with the aim to prepare compounds hopefully characterized by a moderate stability, so as to have a balance between systemic stability and rapid drug release, we attempted to prepare compounds structurally similar to **15** but possessing a shorter spacer. Thus, the known emisuccinate **16**[22] and emiglutarate **17**[22] (Scheme 4), after activation reaction and condensation with **1** under the same conditions used for **13**, gave large amounts of the corresponding imide compounds **18** and **19**, respectively. To gain insights on the degradation pathway of the conjugates **3a**−**c**, **10** and **15**, the main degradation products in buffer and physiological medium were examined by LC−mass spectrometry (LC−MS). Thus, the ESI LC−MS analysis in negative mode of the mixture obtained from the stability studies in physiological medium of compound **3a** after 24 h showed the presence of the Ara-C (*m*/*z* 268.7) and the imidazopyridinemethyl ion (*m*/*z* 309) but no trace of the starting material **3a** (*m*/*z* 578). In contrast, the ESI LC−MS analysis in negative mode for **10** and **15** after 24 h showed the presence of the Ara-C (*m*/*z* 268.7)

Scheme 3[a]



[a] (a) 4-Nitrophenyl chloroformate, anhydrous THF, TEA; (b) DMF, TEA.

and the starting imidazopyridines **8** (*m*/*z* 419) and **11** (*m*/*z* 384), respectively.

Affinities of Imidazopyridine Derivatives for Peripheral and Central Benzodiazepine Receptors. The affinities of the conjugates **3a−c**, **10** and **15** for CBR and TSPO were evaluated by measuring their ability to compete with [^{3}H]flunitrazepam and [^{3}H]PK 11195 binding, respectively, to membrane preparations from rat cerebral cortex. Their affinities were compared with those of unlabeled PK 11195. The measured binding affinities for CBR and TSPO are shown in Table 2.

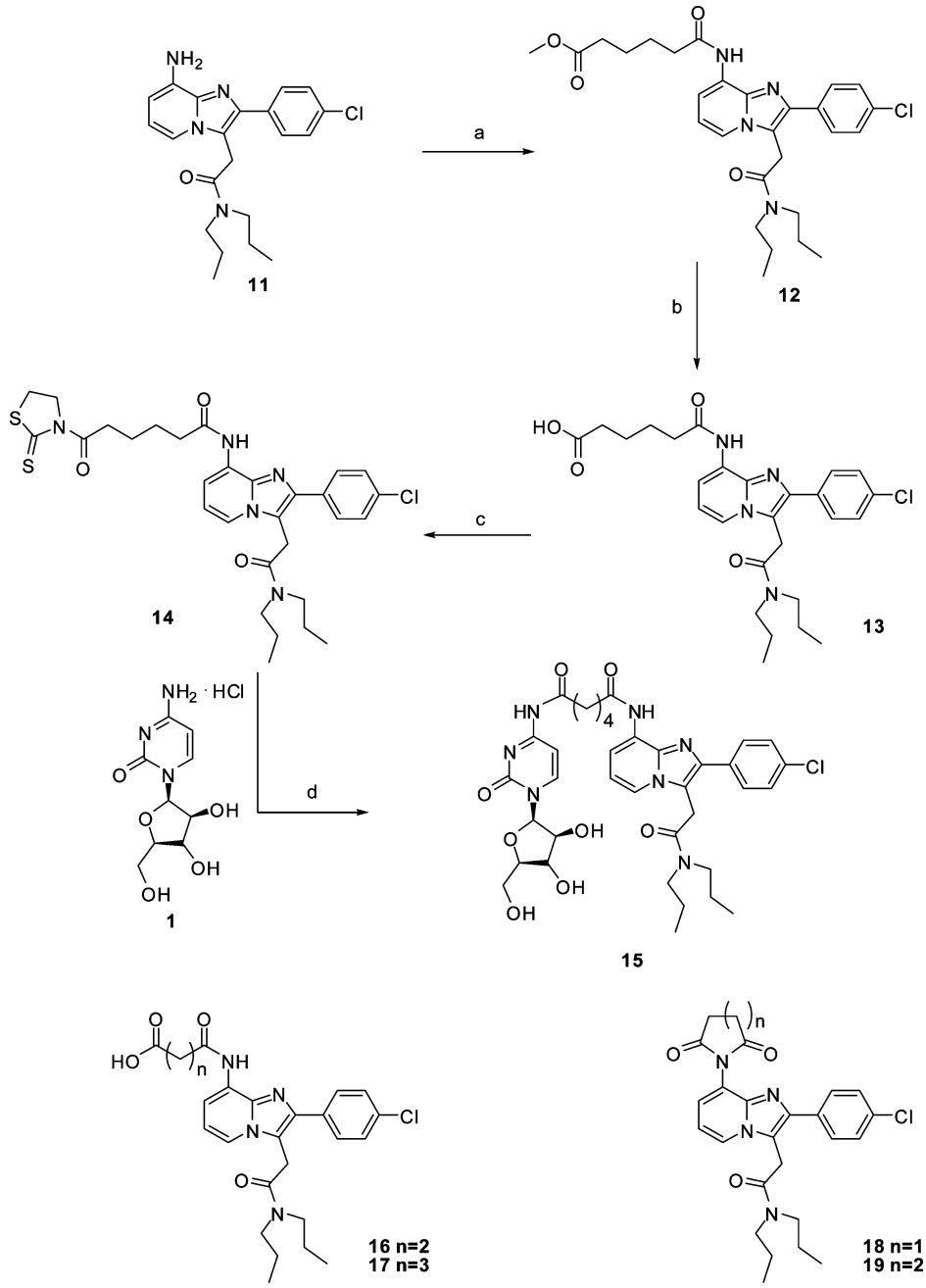
The analysis of the binding affinities of the entire set of compounds **3a−c** indicated that conjugates possessed low selectivity and affinity, being in the micromolar range for TSPO. Unlike conjugates **3**, compounds **10** and **15** showed high affinity and selectivity for TSPO (2.22 nM and 2.13 nM, respectively, Table 2).

Cytotoxicity Studies. The results of the cytotoxicity assays of the Ara-C conjugates **3a−c**, **10** and **15** conducted against glioma cancer cells are also shown in Table 2. In this regard, it should be noted that TSPO expression has been well characterized in glioma cancer cell lines.[43] The SF126 glioma cell line was the most resistant to the conjugates **3a−c**, while compounds **10** and **15** resulted active even though less than Ara-C (Table 2). The high TSPO affinity and selectivity observed for conjugate **15** coupled with its relevant stability in both phosphate buffer and serum supported an evaluation of its cytotoxicity profile against glioma cell lines expressing high levels of TSPO. In particular, conjugate **15** was tested against SF188, RG2 and C6 glioma cells, and the results observed are also reported in Table 2. As can be seen, conjugate **15** resulted *in vitro* once again less cytotoxic than Ara-C against these glioma cell lines. However, the appreciable lipophilicity of **15** could enhance the BBB penetration by passive diffusion independently of NT proteins and, hence, its administration may overcome drug resistance in cancer cells with a deficiency in NT.[33] In addition, since the stable conjugate **15** is characterized by high affinity and selectivity for TSPO, it may possess enhanced propensity to target tumor cells *in vivo*. Both these features of the Ara-C derivative **15** could be advantageous to improve the clinical potential of this nucleoside drug. Therefore, we considered it of interest to evaluate the cytotoxicity of **15** and Ara-C against C6 glioma cells in the presence of potent NT inhibitors as well as to assess the transport properties across an *in vitro* BBB model of the conjugate.

*Cytotoxicity Assays of Conjugate **15** and Ara-C against C6 Glioma Cells in the Presence of Nucleoside Transport Inhibitors.* The effects of compound **15** and Ara-C on C6 glioma cells were evaluated in the presence or absence of the well-known NT inhibitors *S*-(4-nitrobenzyl)-6-thioinosine

(43) Yamasaki, T.; Kumata, K.; Yanamotoa, K.; Hatori, A.; Takei, M.; Nakamura, Y.; Koike, S.; Ando, K.; Suzuki, K.; Zhang, M. R. Imaging of peripheral-type benzodiazepine receptor in tumor: in vitro binding and in vivo biodistribution of *N*-benzyl-*N*-[^{11}C]methyl-2-(7-methyl-8-oxo-2-phenyl-7,8-dihydro-9H-purin-9-yl)acetamide. *Nucl. Med. Biol.* **2009**, *36*, 801–809.

Scheme 4[a]



[a] (a) Methyl adipoyl chloride, K_2CO_3, anhydrous THF; (b) 100 nM NaOH/1,4-dioxane, 50° C; (c) EDC, DMAP, 2-mercaptothiazoline, CH_2Cl_2; (d) anhydrous pyridine, 50° C.

(NBTI) and dipyridamole at subtoxic concentrations of 100 μM and 30 μM, respectively.[44] The IC_{50} values of compound **15** and Ara-C, presented in Table 3, were 1.28 ± 0.06 and 0.005 ± 0.001 μM, respectively. Thus, the presence of the transport inhibitors did not reverse the cytotoxic activity of compound **15**, while the cytotoxicity of Ara-C was reduced of 1300−1400-fold by dipyridamole, whereas the inhibitory effect of Ara-C on cell proliferation was reduced 200−300-fold by NBTI. These results clearly demonstrated that the transport of compound **15** is not affected by the presence of NT inhibitors and, moreover, it may overcome the drug resistance to Ara-C due to deficient NT systems.

In Vitro **P-Glycoprotein Assays To Predict the Interaction of P-gp with 15.** It is important to establish if a compound interacts with P-gp as a substrate, a modulator, or an inhibitor. A wide range of methodologies have been used to characterize drug interaction with P-gp. These

(44) Breistøl, K.; Balzarini, J.; Sandvold, M. L.; Myhren, F.; Martinsen, M.; De Clercq, E.; Fodstad, Ø. Antitumor activity of P-4055 (elaidic acid-cytarabine) compared to cytarabine in metastatic and s.c. human tumor xenograft models. *Cancer Res.* **1999**, *59*, 2944–2949.

Table 1. Lipophilicity and Chemical and Enzymatic Stability of Conjugates **3a**−**c**, **10**, **15** and Ara-C

		$t_{1/2}$[b] (h)	
compd	CLOGP[a]	phosphate buffer 0.05 M, pH 7.4	diluted serum
3a	2.72	13.5	4.5
3b	2.00	16.4	2
3c	2.00	30	4.6
10	3.34	0.5	*c*
15	2.84	144	18
Ara-C	−2.19		

[a] Estimated according to ChemDraw Ultra 10.0 software. [b] Based on the loss of starting material. [c] Unstable.

methods can employ intact cells or purified protein, and a combination of different approaches are often required to identify the mechanism of interaction. A number of P-gp expressing cell lines can be used to assess the interaction and transport of new chemical entities with the protein including Caco-2 and MDCK. The Caco-2 cell line is well-known to display high levels of P-gp, which plays an important role in the efflux of drugs. Therefore, at first we evaluated whether conjugate **15** is substrate, modulator or inhibitor of P-gp using the calcein AM fluorescent, the ATPase assays and the bidirectional transport study on Caco-2 cell lines.[39] Calcein AM is a lipophilic MDR1 and MRP1 substrate able to cross the cell membrane. In the cell compartment it is hydrolyzed by endogenous cytoplasmic esterases yielding highly fluorescent calcein, which, in turn, is not a MDR1 and MRP1 substrate, and it cannot cross the cell membrane *via* passive diffusion. Thus, a rapid increase in the fluorescence of cytoplasmic calcein can be monitored. As reported in Table 4, the observed IC_{50} value for **15** in the calcein AM assay was 67 μM. In the P-gp ATPase activity assay, drug-stimulated P-gp ATPase activity was estimated by measuring the level of inorganic phosphate released from ATP. In this assay it was found that conjugate **15** was unable to deplete ATP. Caco-2 monolayer grown on permeable filters are commonly employed to characterize P-gp substrates. The P_{app} values in both apical to basolateral (P_{app}, AP) and basolateral to apical (P_{app}, BL) directions on Caco-2 monolayer were used to evaluate the BL/AP ratio. In these experiments carried out on conjugate **15**, the BL/AP ratio was found to be 9.6.

*Transport Studies on **15** through MDCKII-MDR1 Monolayer.* To obtain information on the ability of compound **15** to cross the BBB, transport studies involving MDCKII-MDR1 monolayer were carried out. This approach constitutes a well established *in vitro* method to estimate drug permeability across the BBB.[45,46] On the other hand, MDCKII-MDR1, as Caco-2 cell lines, are well-known to overexpress P-gp. In these studies, the formation of confluent MDCK monolayer with functional tight junctions was confirmed by microscopy, TEER values and flux of FD4 and diazepam. The transport was followed for 3 h and the average MDCK TEER value was approximately 800 Ω/cm^2. The results reported in Table 4 indicate that compound **15** is characterized by P_{app} value greater than the paracellular marker FD4 and Ara-C as well but lower than that of the transcellular marker diazepam. In particular, compound **15** P_{app} value resulted 7.8 times greater than free Ara-C and, in agreement with its lipophilic character (Table 1, CLOGP 2.84), it should overcome the BBB by the transcellular pathway. In addition, it should be noted that when the transport experiments on **15** were repeated in the presence of well-known P-gp inhibitors, such as verapamil and elacridar, no significant difference in P_{app} values was observed.

Discussion

The main aim of the present work was to synthesize TSPO ligand−AraC conjugates and to evaluate their *in vitro* receptor binding, chemical stability, cytotoxic and transport properties. For this purpose, the TSPO ligand−Ara-C conjugates **3a**−**c**, **10** and **15** were designed and prepared. In particular, in conjugates **3a**−**c** the hydrophilic Ara-C moiety was introduced on the amide nitrogen at the 3-position of the imidazopyridine nucleus, while the same moiety was linked through appropriate spacers at 8- and at the *para*-position of the 2-phenylimidazopyridine skeleton for **15** and **10**, respectively. On the other hand, it must be noted that **3a**−**c**, **10** and **15**, as N^4-acyl derivatives of Ara-C, should display a metabolic stability against the cytosine nucleoside deaminase greater than the parent drug. In fact, although acylation of the *N*-amino group of Ara-C is known to be a difficult task due to the low nucleophilicity of the aromatic NH_2, N^4 derivation of Ara-C has been shown to prevent inactivation by cytidine deaminase.[36] In the case of **15** the obstacle of the low nucleophilicity of the aromatic NH_2 was efficiently overcome by employing the intermediate acylthiazolidinethione **14**, which is remarkably reactive and selective toward the N^4-amino group.

The differences in binding affinity and selectivity observed for the conjugates herein studied are consistent with the structure−affinity relationship analysis[22] of the 2-phenylimidazo[1,2-*a*]pyridine derivatives that suggested the substitution with three chlorine atoms on the imidazopyridine nucleus would lead to a favorable interaction with the corresponding complementary site of the receptor. However, it has been pointed out that introduction of hydrophilic substituents (such as Ara-C moiety) at the 8- and at the *para*-position of the 2-phenylimidazopyridine skeleton leads to compounds endowed with high affinity and selectivity, and it can explain the observed high receptor binding affinity and selectivity of conjugates **10** and **15**, whereas the presence of hydrophilic substituents on the amide nitrogen at the 3-position of the

(45) Garberg, P.; Ball, M.; Borg, N.; Cecchelli, R.; Fenart, L.; Hurst, R. D.; Lindmark, T.; Mabondzo, A.; Nilsson, J. E.; Raub, T. J.; Stanimirovic, D.; Terasaki, T.; Öberg, J. O.; Österberg, T. In vitro models for the blood-brain barrier. *Toxicol. in Vitro* **2005**, *19*, 299–334.

(46) Mensch, J.; Oyarzabal, J.; Mackie, C.; Augustijns, P. In vivo, in vitro and in silico methods for small molecule transfer across BBB. *J. Pharm. Sci.* **2009**, *98*, 4429–4468.

Table 2. Affinity for Rat Cerebrocortical CBR and TSPO and Cytotoxicity against Glioma Cells of Compounds **3a**−**c**, **10**, **15** and Ara-C

	receptor binding assays: IC_{50} (nM)[a]		cytotoxicity assays: IC_{50} (μM)[a]			
compd	CBR	TSPO	SF126	SF188	RG2	C6
3a	(24%)[b]	(45%)[b]	IS[c]			
3b	2480	(20%)[b]	IS			
3c	6140	(27%)[b]	IS			
10	>10000	2.22[d]	6.90 ± 3.79			
15	>10000	2.13[d]	10.00 ± 2.77	1.73 ± 0.85	3.00 ± 0.90	1.37 ± 0.64
Ara-C	*e*	*e*	1.25 ± 0.07	0.003 ± 0.002	0.003 ± 0.003	0.005 ± 0.001

[a] Data are means ± SD of three separate experiments performed in duplicate (CV < 10%) and are reported in nM, unless otherwise expressed. [b] Values in parentheses are the percentages of inhibition of specific [^{3}H]flunitrazepam binding determined at 40 μM concentration of the tested compound and of specific [^{3}H]PK11195 at 1×10^{-5} M for CBR and TSPO affinity, respectively. [c] IS, insensitive. [d] For comparison purpose the IC_{50} of the reference TSPO ligand is reported and namely 2.1 nM. [e] No receptor binding affinity, predicted IC_{50} > 10000 nM.

Table 3. Effects of Nucleoside Transport Inhibition on the *in Vitro* Cytotoxic Effect of Cytarabine Hydrochloride and Compound **15** on C6 Glioma Cell Line

	IC_{50} (μM)	
nucleoside transport inhibitor	Ara-C	compd **15**
without inhibitor	0.005 ± 0.001	1.28 ± 0.06
+NBTI[a]	1.07 ± 0.46	0.85 ± 0.05
+dipyridamole[b]	7.08 ± 0.64	1.34 ± 0.02

[a] NBTI was used at a subtoxic concentration of 100 μM. [b] Dipyridamole was used at a subtoxic concentration of 30 μM.

imidazopyridine nucleus was shown detrimental for affinity and selectivity as observed for compounds **3**.[20−22]

As for the results of stability studies, particularly those aimed to gain information on the main degradation products in buffer and physiological medium by LC−MS, it should be pointed out that the analytical approach followed provides only qualitative information. So, the actual degradation pathway(s) is (are) unknown and the extent of the **3a**−**c**, **10** and **15** cleavage at the amide bond level leading to free Ara-C is unknown as well. The marked decrease in cytotoxicity of conjugates should be mainly due to an intracellular uptake of conjugates and 5′-monophosphate activation by deoxycytidine kinase lower than what occurs for the free drug. In fact, Ara-C is able to enter cells by NT, but we do not know if such a process could account for transport of an intact conjugate. The alternative explanation that **3a**−**c**, **10** and **15** toxicity is connected with their hydrolysis to give the free Ara-C is not in agreement with the stability data because it is to be expected the cytotoxicity of the essentially unstable conjugate **10** should be comparable to or even better than that of free drug. In fact, the hydrolysis of compound **10** produces also compound **8**. This TSPO ligand is a proapoptotic agent which, in combination with Ara-C, may cause a synergistic growth inhibition of cancer cells. Such an effect may also occur for compound **15** but not for compounds **3a**−**c**, which led to the formation of compounds **2a**−**c** endowed with weak TSPO affinity and proapoptotic activity. Therefore, the pathway that **3a**−**c**, **10** and **15** toxicity is connected with their hydrolysis to give the free Ara-C, as suggested by the presence of the free drug in the mixture examined by LC−MS, does not satisfactorily explain the cytotoxicity of **10** being much lower than that of Ara-C. On the other hand, the stability of **15** being greater than that of **10** may also account for the lower cytotoxicity of the former against the same glioma cell line. In our opinion, the cytotoxicity data can be rationalized assuming that the trend observed should be mainly due to a different intracellular uptake and activation by cellular enzymes in combination with the hydrolysis pathway to give the free drug, the latter occurring to a lower extent. Definitive conclusions in this regard can be reached after a detailed study on the conjugates' degradation kinetics.

The studies aimed to predict the interaction of P-gp with **15** were motivated by the fact that P-gp serves as a drug efflux pump on the BBB and, possibly, on tumor cells, whereby it may act on lipophilic substrates. A variety of *in vitro* assays have been used to classify compounds as P-gp substrates, modulators or inhibitors. Substrates are molecules actively transported by the protein and therefore have a higher concentration outside the cell with respect to the cytosol. Modulators interact at the binding sites, therefore reducing substrate binding through a negative allosteric interaction. Inhibitors interfere with the substrate thereby blocking P-gp translocation.[47] Modulators and inhibitors exert the same final biological effect, restoring cell sensitivity to chemotherapeutic agents, and have been the subject of numerous investigations.[47] The calcein AM fluorescent, the ATPase assays and the bidirectional transport study on Caco-2 cell line are well established for classification purposes.[39,47] Results from these assays conducted on **15**, taken together, indicate that the conjugate **15** behaves as a clear P-gp modulator.[47] In fact, it has been recently evidenced that a BL/AP ratio from 18 to 20 and a ratio <2 identifies substrates and inhibitors, respectively, while modulators show intermediate ratios ranging from 2 to 18.[47] The BL/AP ratio of 9.6 allow us to classify **15** as a P-gp modulator. On the other hand, the fact that the P_{app} values observed for **15** are

(47) Colabufo, N. A.; Berardi, F.; Cantore, M.; Contino, M.; Inglese, C.; Niso, M.; Perrone, R. Perspectives of P-glycoprotein modulating agents in oncology and neurodegenerative diseases: pharmaceutical, biological, and diagnostic potentials. *J. Med. Chem.* **2010**, *53*, 1883–1897.

Table 4. Transport across MDCKII-MDR1 Cells and on Compound **15** and Evaluation of Its Interaction with P-Glycoprotein by Three Specific Biological Assays

	transport study on MDCKII-MDR1[a]		P-gp interaction specific biological assays[a]		
compd	P_{app} (AP) (cm/s)	P_{app} (BL) (cm/s)	P_{app} (BL/AP)	calcein IC_{50} (μM)	ATPase activation
15	$6.01 \pm 1.23 \times 10^{-6}$ (3.7)[b]	$2.21 \pm 0.48 \times 10^{-5}$	9.6[c]	67[d]	no
Ara-C	$7.72 \pm 1.78 \times 10^{-7}$				
diazepam	$5.09 \pm 0.52 \times 10^{-5}$				
FD4	$2.96 \pm 0.45 \times 10^{-10}$				

[a] Data are means ± SD of three determination. [b] The value in parentheses is the permeability BL/AP ratio. [c] The P_{app} in both AP and BL directions was determined on Caco-2 monolayer. [d] Elacridar, a known inhibitor of P-gp, was used as the positive control (IC_{50} 0.01 μM).

not significantly influenced by the presence of well-known P-gp inhibitors, such as verapamil and elacridar, confirms that this compound is not a P-gp substrate.

In conclusion, synthetic routes toward imidazopyridine-TSPO ligand−Ara-C conjugates **3**, **10** and **15** have been developed. These new compounds displayed variable *in vitro* TSPO binding ranging from low/moderate (**3a**−**c**) to high (**10** and **15**) affinity and selectivity. Receptor binding affinity data combined with the stability results induced us to focus our attention on compound **15**. First of all, as N^4-acyl derivative of Ara-C should be resistant to inactivation by cytidine deaminase. The evaluation of its cytotoxicity profile against further glioma cell lines expressing high levels of TSPO showed an activity less than that of Ara-C. However, in contrast to that observed for free parent drug, the presence of NT inhibitors did not affect the cytotoxic activity of **15**. *In vitro* studies suggest that **15** behaves as a clear P-gp modulator and thereby may be useful to reverse MDR. Transport studies across the MDCKII-MDR1 monolayer indicated that conjugate **15** should overcome the BBB by transcellular pathway. Therefore, based on the *in vitro* properties of conjugate **15**, which consisted of its high affinity and selectivity for TPSO combined and chemical and metabolic stability, its further evaluation *in vivo* as a means to target brain tumors is warranted.

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