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

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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 N4-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 are abundant in peripheral organs like kidney, liver, lung as well as in glial cells of the central nervous system. (1)

system (CNS). 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 within cells and are composed of at least three subunits: the isoquinoline-binding protein, a voltage-dependent anion channel, and an adenine nucleotide carrier. The trimeric complex is a component of the mitochondrial permeability transition (MPT) pore, which plays an important role in the passage of various biogenic compounds between the cytoplasm and mitochondrial intermembrane. There is the passage of various biogenic compounds between the transitional (MPT) pore, which plays an important role in mitochondria and in steroidogenesis as well as in various neurological diseases such as Alzheimer’s disease, Huntington’s disease, epilepsy and stroke-induced brain injury. 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. Moreover, there is evidence indicating TSPO-specific ligands induce apoptosis and cell cycle arrest in cancer cells, 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), isoquinolines (PK-11195), 2-aryl-3-indoleacетamides (FGIN-1–27), and N-phenyl-N-isopropoxybenzyl-acetamides (DAA1097) (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) it is known that malignant brain tumors are among the most challenging to treat, and the limited survival advantage attributed to chemotherapy is primarily due to low CNS penetration.

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. 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, a 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. 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 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.

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. AraC has also been part of combination chemotherapeutic regimens for various brain tumors including primary CNS lymphoma, and recurrent or refractory malignant gliomas. 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., Depocyt).

Ara-C is rapidly converted to an inactive and more soluble metabolite 1-(β-D-arabinofuranosyl)uracil (Ara-U) by cytosine deaminase, which reduces its therapeutic efficacy. To improve the efficacy of Ara-C, delivery systems that selectively target brain tumors and overcome the BBB have been devised to devise TSPO-based drug delivery systems to selectively target brain tumors and overcome the BBB.

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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. The complexities of Ara-C’s membrane transport attributes, rapid clearance, and intracellular activation have promoted many efforts to modify its biological activity and/or to modify its pharmacokinetic properties. In particular, N<sup>4</sup>-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.

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 (HO-BZT), dicyclohexylcarbodiimide (DCC), triethylamine (TEA), 1,1′-carbonyldimidazole (CDI), N-(3-(dimethylaminopropyl))-N′-ethylcarbodiimide (EDC) and 4-(dimethylamino)pyridine (DMAP) were purchased from Sigma-Aldrich (Milan, Italy). The preparation of the imidazopyridinacetamide TSPO ligand—Ara-C conjugates (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.

Apparatus. Melting points were determined in open capillary tubes with a Büchi apparatus and are uncorrected.

Preparation of Conjugates

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-

IR spectra were obtained on a Perkin-Elmer Spectrum one system spectrophotometer (KBr pellets for solid).<sup>1</sup>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, <sup>1</sup>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 pre-column 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 pre-column 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.
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-Dichloro-2-(4-chlorophenylimidazo[1,2-a]pyridin-3-yl)-N-(1-(2R,3S,4R,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-d₆) δ: 3.5–3.7 (m, 2H, 5'-CH₂O Ara-C), 3.7–4.1 (m, 3H, 2', 3',4'-CHO Ara-C), 4.40 (s, 2H, CH₂CO), 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. (C₂₅H₂₅Cl₂N₅O₆) C, H, N.

2-(6-Dichloro-2-(phenylimidazo[1,2-a]pyridin-3-yl)-N-(1-(2R,3S,4R,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, 1712, 1648 cm⁻¹. ¹H NMR (DMSO-d₆) δ: 3.5–3.7 (m, 2H, 5'-CH₂O Ara-C), 3.7–4.1 (m, 3H, 2', 3',4'-CHO Ara-C), 4.41 (s, 2H, CH₂CO), 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 (d, J = 1H, 1H, NHCO). MS (ESI) m/z: 546 [M + H]⁺. Anal. (C₂₅H₂₅Cl₂N₅O₆) C, H, N.

Preparation of (2R,3S,4R,5R)-2-(4-Amino-2-oxopyrimidin-1(2H)-yl)-4-(phenyl)imidazo[1,2-a]pyridin-3-yl)acetamide (2a). 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 CH₃Cl (20 mL) were added HOBt (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.

4. Yield: 48%. Oil. IR (KBr): 3243, 1749, 1648 cm⁻¹. ¹H NMR (CDCl₃) δ: 1.33 (s, 9H, CH₃), 1.44 (s, 18H, CH₃), 4.1–4.2 (m, 1H, 4'-CHO), 4.3–4.5 (m, 2H, 5'-CHO), 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 (CDCl₃) δ: 1.33 (s, 9H, CH₃), 1.44 (s, 18H, CH₃), 4.1–4.2 (m, 1H, 4'-CHO), 4.3–4.5 (m, 2H, 5'-CHO), 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,3S,4R,5R)-2-(4-(tert-Butoxycarbonylaminono)-2-oxopyrimidin-1(2H)-yl)-3,4-bis(tert-butoxycarbonyloxy)tetrahydrofuran-2-yl)methyl2-(6,8-Dichloro-2-(4-chlorophenylimidazo[1,2-a]pyridin-3-yl)acetate (6) and (2R,3S,4R,5R)-2-(4-(6,8-Dichloro-2-(4-chlorophenylimidazo[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 CH₃Cl (20 mL) were added HOBt (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 (CDCl₃) δ: 1.31 (s, 9H, CH₃), 1.50 (s, 9H, CH₃), 1.51 (s, 9H, CH₃), 4.11 (s, 2H, CH₂CO), 4.2–4.7 (m, 4H, 5'-CHO₂ 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): 3422, 1736, 1652 cm⁻¹. ¹H NMR (CDCl₃) δ: 1.42 (s, 9H, CH₃), 1.44 (s, 9H, CH₃), 1.48 (s, 9H, CH₃), 3.8–4.0 (m, 2H, CH₂CO), 4.2–4.6 (m, 4H, 5'-CHO₂ 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 CH₂Cl₂ (10 mL) at rt was added TFA (5 mL), and the reaction progress was monitored by TLC (CHCl₃/CH₃OH 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-dihydroimidazin-4-ylcarbamate (10). Yield: 23%. Mp: 156 °C dec. IR (KBr): 3327, 1704, 1627 cm⁻¹. ¹H NMR (DMSO-d₆): δ: 0.7–0.9 (m, 6H, CH₃), 1.4–1.7 (m, 4H, CH₃), 3.1–3.4 (m, 4H, -CH₂NCO), 3.1–3.4 (m, 2H, 5′-CH₂O Ara-C), 3.8–4.1 (m, 3H, 2′, 3′, 4′-CHO Ara-C), 4.28 (s, 2H, -CH₂CON), 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. (C₃₉H₂₅Cl₂N₉O₅) C, H, N.

Procedure for the Preparation of Conjugate 15. A mixture of compound 13 (0.36 mmol), 2-mercaptoethanol (0.43 mmol), EDC·HCl (0.39 mmol), and DMAP (0.43 mmol) in anhydrous CH₂Cl₂ (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 K₂CO₃ (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 Na₂SO₄ 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 (CDCl₃): δ: 0.7 (t, J = 7.4 Hz, 3H, CH₃), 1.3–1.4 (m, 4H, CH₂), 1.6–2.0 (m, 4H, CH₂), 2.37 (t, J = 7.15 Hz, 2H, CH₂CON), 2.53 (t, J = 7.15 Hz, 2H, CH₂COO), 3.11 (t, J = 7.4 Hz, 2H, CH₂NCO), 3.28 (t, J = 7.4 Hz, 2H, CH₂NCO), 3.67 (s, 3H, CH₃O), 4.05 (s, 2H, CH₂CON), 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. (C₂₉H₂₃Cl₂N₂O₄) C, H, N.
The experimental protocol was approved by the Animal Ethics 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.

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 a water bath at constant temperature of 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 residual starting material against time.

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.

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.

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 \(\sim 10,000\) 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 dipyriramole (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 (\(P_{\text{app}}, \text{AP}\)) and basolateral to apical (\(P_{\text{app}}, \text{BL}\)) 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 measurements. The apparent permeability, in units of nm/s, was calculated using eq 1.

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

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 MDRI 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 \(\sim 50,000\) 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 \(2 \times 10^4\) 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 Transwell flat-bottom 24-well plate. Medium in the top well (100 µL) was replaced with the same volume of fresh complete medium and the experiments were repeated three times. Apical to basolateral (\(P_{\text{app}}, \text{AP}\)) and basolateral to apical (\(P_{\text{app}}, \text{BL}\)) 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 measurements. The apparent permeability, in units of nm/s, was calculated using eq 1.

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

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

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.

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, 1H NMR, mass spectra, and elemental analyses. An interesting feature of the 1H NMR spectra of the Ara-C-imidazooacetamides 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 (Δδ 1.04 ppm and 0.15 ppm, respectively). This may be attributed to the deshielding effect of the N3-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 alkalization 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.


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 CH₂Cl₂ 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 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 with methyl adipoyl chloride in the presence of K₂CO₃ to give the ester 12, which was hydrolyzed to the corresponding acid 13. To obtain the selective amidification of the N⁴-amino group of Ara-C 1, compound 13 was first activated with 2-mercaptothiazoline to yield the active thiazolidine thione 14 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 and emiglutarate 17 (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)
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 [3H]flunitrazepam and [3H]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.

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(NBTI) and dipyridamole at subtoxic concentrations of 100 µM and 30 µM, respectively. The IC₅₀ 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

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(Scheme 4)

(a) Methyl adipoyl chloride, K₂CO₃, anhydrous THF; (b) 100 nM NaOH/1,4-dioxane, 50°C; (c) EDC, DMAP, 2-mercaptothiazoline, CH₂Cl₂; (d) anhydrous pyridine, 50°C.

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

<table>
<thead>
<tr>
<th>compd</th>
<th>CLOGP*</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>phosphate buffer 0.05 M, pH 7.4</th>
<th>diluted serum</th>
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<td>3a</td>
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<td>3b</td>
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<tr>
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<td>30</td>
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</tr>
<tr>
<td>10</td>
<td>3.34</td>
<td></td>
<td>0.5</td>
<td>c</td>
</tr>
<tr>
<td>15</td>
<td>2.84</td>
<td></td>
<td>144</td>
<td>18</td>
</tr>
<tr>
<td>Ara-C</td>
<td>–2.19</td>
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* Estimated according to ChemDraw Ultra 10.0 software.

**Based on the loss of starting material.**

**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 ATPase inhibitors of P-gp using the calcein AM fluorescent, the MDCK cell line, 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 \(P_{\text{app}}\) 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 \(P_{\text{app}}\) 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 \(P_{\text{app}}\) values was observed.

**Discussion**

The main aim of the present work was to synthesize TSPO ligand–Ara-C 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-phenylimidazol[1,2-\(\alpha\)]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


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 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 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.

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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 N4-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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