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Design, synthesis and in vitro evaluation on HRPE cells of ascorbic and 6-bromoascorbic acid conjugates with neuroactive molecules

Stefano Manfredini,[a,*] Silvia Vertuani,[a] Barbara Pavan,[b] Federica Vitali,[a] Martina Scaglianti,[a] Fabrizio Bortolotti,[a] Carla Biondi,[b] Angelo Scatturin,[a] Puttur Prasad[c] and Alessandro Dalpiaz[a]

[a]*Department of Pharmaceutical Sciences, via Fossato di Mortara 17–19, I-44100 Ferrara, Italy*
[b]*Department of Biology, General Physiology Section, via Borsari 46, I-44100 Ferrara, Italy*
[c]*Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta, GA, USA*

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Abstract—Preliminary investigations allowed us to anticipate that conjugation of nipecotic acid with L-ascorbate (AA) gave a prodrug endowed with anticonvulsant activity in mice. In view of these results, and in order to get deepen insight into the molecular aspects at the base of the transport mechanism, a second generation of compounds, based on 6-bromo-6-deoxy-L-ascorbic acid (BrAA) as the carrier molecule was designed and synthesized. Effects of the chirality of the transported drug was also investigated on *R*- and *S*-nipecotic acid. Interaction and uptake modalities were evaluated in our in vitro model based on human retinal pigment epithelium cells (HRPE), which expresses the membrane L-ascorbic acid (AA) SVCT2 transporters. A remarkable increase on SVCT2 affinity was found going from AA to BrAA conjugates, that is, **11** ($K_i = 1187 \pm 78\,\mu M$) versus **19** ($K_i = 193 \pm 14\,\mu M$) and **12** ($K_i = 39.8 \pm 3.2\,\mu M$) versus **20**, ($K_i = 7.4 \pm 0.8\,\mu M$). Taken together, these data are in agreement with our initial hypothesis on the possibility to achieve better affinities by conjugation with AA analogs, and also consent to hypothesize the presence of accessory interactions that may improve transporters recognition.

1. Introduction

Central nervous system (CNS) treatments are one of the major trust in the pharmaceutical industry but, to take advantage of many powerful new therapies, the barriers to the brain must be reckoned with.[1] In fact, the diffusion of neuropharmaceutical agents from the blood into the brain is complicated by the presence of two physiological barriers: the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB). The BBB is maintained by the endothelial tight junctions within the brain microvasculature.[2,3] The BCSFB is located at the choroid plexuses and is formed by epithelial cells held together at their apices by tight junctions.[4]

Many different strategies have been proposed as a way to deliver a compound from the blood to the brain, ranging from invasive approaches to mildest methods that involve the modification of the physicochemical properties of drugs.[4]

This latter approach has been largely explored in terms of improving drug BBB penetration by increase of the overall lipophilicity of the molecule by either reducing the relative number of polar groups, masking them with lipid carriers and/or attaching lipid moieties to the molecule.[3] These approaches, however, were usually accompanied by several side processes that influence distribution such as rapid elimination, peripheral distribution and binding to plasma proteins.[3,5,6] Recently, the progress of molecular cloning and expression of genes related to endogenous transporters, suggested the synthesis of prodrugs able to interact with specific transporters of native compounds.[3] Several specific transporters have been identified in boundary tissues

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* Corresponding author. Tel.: +39 0532 291292; fax: +39 0532 291296; e-mail: mv9@dns.unife.it

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between blood and CNS compartments. Some of them are involved in the active supply of nutrients (i.e., glucose, amino acids, vitamins) and have been used to explore prodrugs with improved brain delivery.[7–10] According to this point of view, we have recently proposed that drug-conjugation with L-ascorbic acid (AA) can improve the delivery into the brain, via interaction with the SVCT2 ascorbate transporters.[10]

SVCT1 and SVCT2 are sodium-dependent transporters of AA,[11,12] which is an essential nutrient that humans need but are not able to synthesize.[13,14] As a consequence, the importance of these human transporters is of fundamental significance to maintain the necessary intake:[15] SVCT1 is responsible for AA absorption from intestine and of the recovery by kidney, whereas SVCT2, expressed by neuroepithelial cells of the choroids plexus and the retinal pigmented epithelium, allows concentration in the brain and eye. Brain, spinal cord and adrenal glands have the highest ascorbate concentrations, this implies a function beyond its action as a cell-specific enzyme co-factor.

During a preliminary investigation,[10] on transport capability of SVCT2 transporters, 6-deoxy-6-bromo-L-ascorbic acid (BrAA) displayed better affinity for the transporters than the natural ligand AA. Moreover, we observed that nipecotic acid, a well known antiepileptic drug, that which poorly permeate the BBB,[16] became able of in vivo anticonvulsant effects when conjugated to AA. These effects were paralleled by good in vitro affinity for SVCT2 transporters. During the same investigation we observed that two other drugs, kynurenic and diclofenamic acids, also became able to interact with SVCT2 transporters upon ascorbate conjugation.

According to these informations, and considering the needs of further studies on the SVCT2 mediated transport into the brain, we have undertaken the present study in order to: (i) prepare and evaluate the corresponding BrAA conjugates of the selected drugs; (ii) prepare and evaluate the two diastereoisomers of 6-nipecotyl-ascorbate, in order to explore possible effects due to the chirality; (iii) investigate in detail the behaviour towards the SVCT2 transporters of selected drugs and related prodrugs obtained by conjugation with AA and BrAA.

Suitable drugs, to be used as model molecule, (Fig. 1) were nipecotic acid, proposed as a potential anticonvulsant,[16] kynurenic acid, proposed for the potential control of neurodegenerative disorders;[17] and diclofenamic acid, proposed for potential application in Alzheimer's diseases.[18–20] The biological activity was investigated by in vitro studies involving the human SVCT2 isoform[21] selectively expressed by human retinal pigment epithelium (HRPE) cells.[10] Finally the experimental procedures previously only communicated will be described in detail.

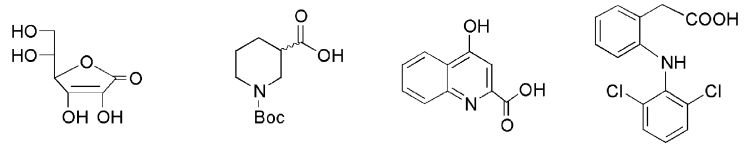
2. Results

2.1. Chemistry

The synthetic approach has taken into account the needing to select positions on L-ascorbic acid (5-(*R*)-[(*S*)-1,2-dihydroxy-ethyl]-3,4-dihydroxy-5*H*-furan-2-one, AA) and 6-bromo-6-deoxy-L-ascorbic acid (5-(*R*)-[2-bromo-1-(*S*)-dihydroxy-ethyl]-3,4-dihydroxy-5*H*-furan-2-one, BrAA) structure, different from those probably involved in the interaction with the transporter. Thus, on a tentative base, and taking into account that 3,4-dehydroascorbic acid does not interact with the transporters,[22] we considered the intact free hydroxyl groups at position 3 and 4 as a prerequisite for the interaction with SVCT2. Thus, esters at 6 and 5 positions on AA and at 5 position on BrAA have been considered. The ester linkage was selected in view of the needing of a reversible type of bond between AA, BrAA and conjugated drugs.

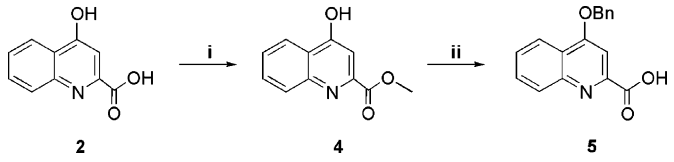
Nipecotic and kynurenic acids needed previous protection at the amino and hydroxyl functions, respectively. Thus nipecotic acid was protected as *tert*-butoxycarbonyl derivative (Boc) (**1**)[23] and kynurenic acid as benzyl derivative (**5**) (Scheme 1). This required prior conversion to the kynurenic acid methyl ester **4**[24] simply accomplished in MeOH under acidic catalysis rather than anhydrous HCl as described previously.

2.1.1. Preparation of AA conjugates. Esterification on AA was carried out by a general procedure involving a protected precursor, namely the 2,3-dibenzyl derivative **6**,[25] in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) (Scheme 2). Mixtures of 6- and/or 5- and/or 5,6-di-*O*-ascorbates were obtained (**7a**–**c**, **8a**,**b**, **9a**–**c**) with large predominance of the 6-isomers over the other products. Structural attributions were based on ^{1}H NMR data, selecting the C2–H furan as diagnostic proton. In particular a deshielding effect was observed in the case of the 5-*O*-ascorbates, in comparison with the corresponding 6-*O*-ascorbates positional isomers. This occurrence was confirmed also in the case of the 6-bromo-5-*O*-ascorbates. The 6-*O*-ascorbates **7a**, **8a**, **9a** were

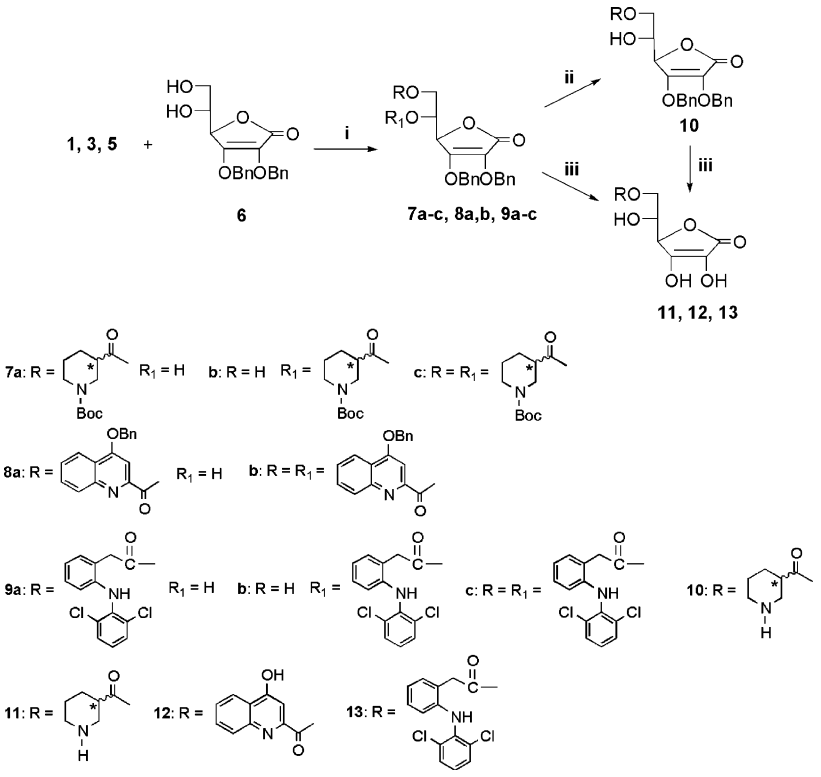


Ascorbic Acid Boc-Nipecotic acid (**1**) Kynurenic acid (**2**) Diclofenamic acid (**3**)

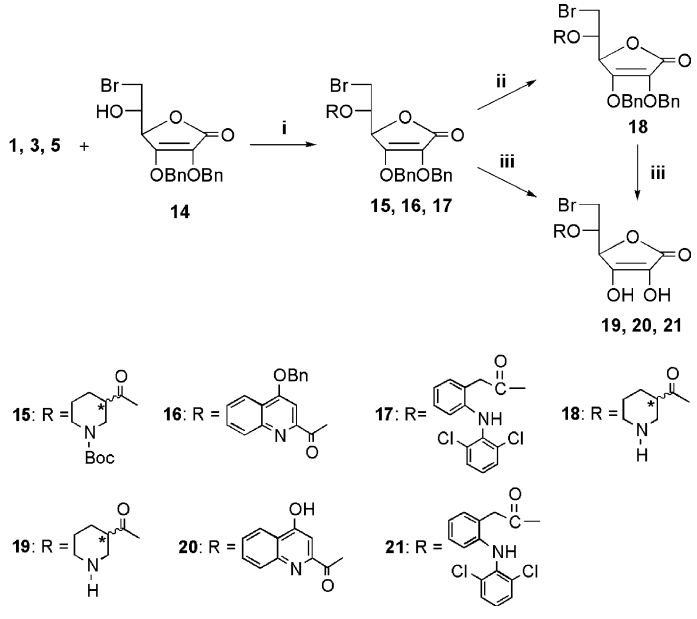
Figure 1. Model drugs used in the study.



Scheme 1. Reagents and conditions: (i) H_2SO_4 96%, anhydrous MeOH, reflux; (ii) (a) benzylbromide, K_2CO_3, anhydrous DMF, (b) NaOH, H_2O/MeOH (1:1).



Scheme 2. Reagents and conditions: (i) DMAP, DCC, anhydrous CH_2Cl_2 or CH_2Cl_2/DMF; (ii) TFA, anhydrous CH_2Cl_2; (iii) H_2, C/Pd 10%, CH_3OH.



Scheme 3. Reagents and conditions: (i) DMAP, DCC, anhydrous CH_2Cl_2 or CH_2Cl_2/DMF; (ii) TFA, anhydrous CH_2Cl_2; (iii) H_2, C/Pd 10%, CH_3OH.

then deprotected by hydrogenolysis (**11**–**13**). In the case of **7a**, prior treatment with trifluoroacetic acid (TFA) was required to remove the Boc-protective group (**10**). The same synthetic strategy used for (*R*,*S*)-nipecotic acid derivatives (**7a**), was applied for the preparation of the corresponding (3*R*,2*S*,2*R*) **7a** and (3*S*,2*S*,2*R*) **7a**.

2.1.2. Preparation of BrAA conjugates. Because of BrAA was endowed with better SVCT2 affinity as compared to AA, BrAA derivatives of nipecotic, kynurenic and diclofenamic acids were prepared as second generation compounds. To this end BrAA was prepared following a literature procedure[26] and the corresponding 3,4-dibenzyl derivative (**14**) was then obtained.[27] After esterification with BrAA in the presence of DCC (Scheme 3) the 6-bromo-6-deoxy-5-*O*-ascorbates **15**–**17** were obtained in satisfactory yields (60%). Next deprotection, as for AA derivatives, gave final **19**, **20** and **21**.

2.2. Biology

The kinetic of the AA uptake mediated by hSVCT2 is described in Figure 2. The rate was found to be hyperbolically related to AA concentration, indicating saturability of the uptake process. The Michaelis–Menten constant (K_t) and V_{max} values for the transport process referred to ascorbate alone were $36 \pm 3\,\mu M$ and $4.3 \pm 0.3\,nmol/10^6$ cells/60 min, respectively. In a previous communication we anticipated that AA conjugates with nipecotic (AA–Nipec) and kynurenic (AA–Kynur) acids were competitive inhibitors of the SVCT2 mediated uptake of AA (Fig. 2).[10]

The inhibition of this transport by the BrAA conjugates of nipecotic (BrAA–Nipec) and kynurenic (BrAA–Kinur) acids are also reported in Figure 2. As it can be observed in Figure 3, the presence of these BrAA conjugates inhibits the AA uptake, without influence on the V_{max}, but causing an increase of K_i value. This behaviour indicates that these conjugates are competitive inhibitors of the SVCT2 mediated uptake of AA as found also for AA–Nipec and AA–Kynur conjugates.[10] Figure 4 reports the inhibition of 50 μM [^{14}C]AA uptake in the presence of varying concentrations of inhibitors. As it can be observed, nipecotic acid was not able to inhibit the [^{14}C]AA uptake in the concentration range investigated (0.5–5000 μM), whereas after conjugation with AA it became able to interact with SVCT2 transporter. The affinity for SVCT2 of the conjugate with nipecotic acid was not significantly affected by chirality: as reported in Table 1, the K_i values of AA–(*R*)-Nipec ($1080 \pm 74\,\mu M$) and AA–(*S*)-Nipec ($1478 \pm 83\,\mu M$) appear similar to the K_i value of AA–(*R*,*S*)-Nipec ($1187 \pm 78\,\mu M$) (Table 1). It is important to note in Figure 4 that diclofenamic acid (**3**) was able itself to strongly inhibit the [^{14}C]AA uptake, showing a K_i value ($3.35 \pm 0.16\,\mu M$) one order of magnitude higher with respect to the natural ligand, AA. A similar behaviour has been also observed for the corresponding BrAA derivative. Moreover, the conjugation with AA allowed to increase the diclofenamic acid affinity for SVCT2 (AA–Diclo K_i value = $0.19 \pm 0.01\,\mu M$), whereas

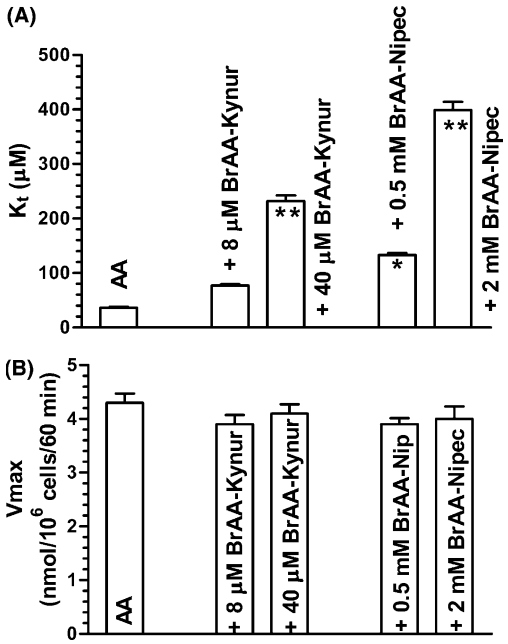


Figure 3. K_t and V_{max} values of AA transport obtained in the absence and in the presence of increasing concentrations of the BrAA conjugates of nipecotic and kynurenic acids. The reference values for AA alone are $K_t = 36 \pm 3\,\mu M$ and $V_{max} = 4.3 \pm 0.3\,nmol/10^6$ cells. $^*p < 0.05$, $^{**}p < 0.001$ significant versus [^{14}C]AA alone.

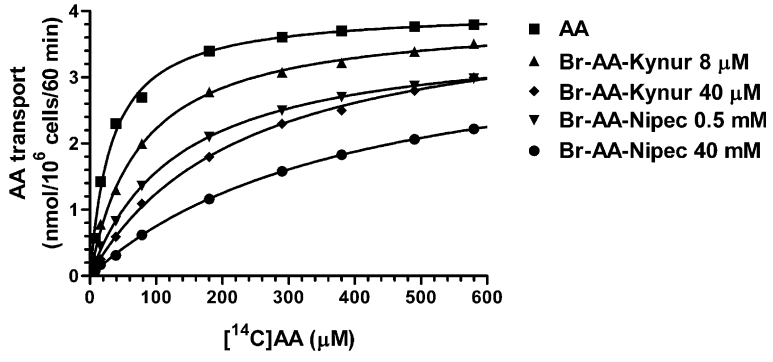


Figure 2. Kinetics of AA uptake in HRPE and inhibition of this transport by different concentrations of BrAA conjugates of nipecotic and kynurenic acids. These are single representative experiments performed in duplicate.

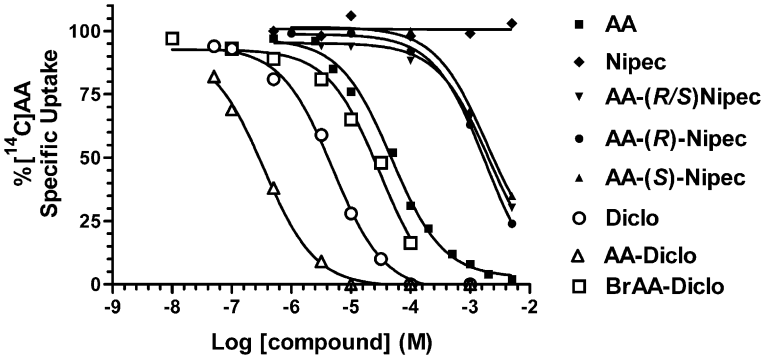


Figure 4. Inhibition of 50 μM [^{14}C]AA uptake by AA, nipecotic and diclofenamic acids and their AA and BrAA-conjugates. These are single representative experiments performed in duplicate.

Table 1. Inhibition constant values (K_i) of AA, BrAA, nipecotic, kynurenic, diclofenamic acids and their conjugates with AA and BrAA, obtained by inhibition of 50 μM [^{14}C]AA uptake on HRPE cells or by Lineweaver–Burk analysis

Compound	K_i (μM)
AA	20.1 ± 1.6[a]
BrAA	2.69 ± 0.13[a]
Nipecotic acid	No interaction[a]
AA–(*R*,*S*)-Nipec	1187 ± 78[a]
AA–(*R*,*S*)-Nipec	1164 ± 99[b]
AA–(*R*)-Nipec	1080 ± 74[a]
AA–(*S*)-Nipec	1478 ± 83[a]
BrAA–(*R*,*S*)-Nipec	192 ± 18[b]
Kynurenic acid	No interaction[a]
AA–Kynur	52.1 ± 4.6[b]
BrAA–Kynur	7.1 ± 1.8[b]
Diclofenamic acid	3.35 ± 0.16[a]
AA–Diclo	0.19 ± 0.01[a]
BrAA–Diclo	21.4 ± 1.8[a]

The conjugate with nipecotic acid has been evaluated either as a 1:1 mixture (AA–(*R*,*S*)-Nipec) or single diasteroisomers (AA–(*R*)-Nipec and AA–(*S*)-Nipec.

[a] K_i values obtained by inhibition experiments.

[b] K_i values obtained by Lineweaver–Burk analysis.

its conjugation with BrAA caused the decrease in potency (BrAA–Diclo K_i value = 21.4 ± 1.8 μM). In contrast with this latter finding, the Lineweaver–Burk analysis of data, reported in Figure 2, indicate that the BrAA conjugation of nipecotic acid increased its affinity towards SVCT2 transporter, with respect to the AA conjugation. The K_i values reported in Table 1 are in fact 1164 ± 99 μM for AA–(*R*,*S*)-Nipec and 192 ± 18 μM for BrAA–Nipec. A similar behaviour was also found for kynurenic acid conjugates with K_i values of 52.1 ± 4.6 μM for AA–Kynur and 7.1 ± 1.8 μM for BrAA–Kynur (Table 1).

3. Discussion

We have previously discovered that conjugation with AA of model drugs, that do not normally cross BBB, gave compounds able to reach CNS.[10] We proposed, using a model based on human HRPE cells, an interaction mediated by human AA SVCT2 transporters at the base of the observed delivery. During this study, human HRPE cells were also proposed as a good cellular model for in vitro studies of the endogenous activity of SVCT2 transporters. In fact, RT-PCR analysis demonstrated that SVCT2 but not SVCT1 isoforms are expressed in human HRPE cells. As previously anticipated,[10] the kinetic studies for [^{14}C]AA confirmed the existence of a saturable transport (Fig. 1). The K_t and V_{max} values obtained for AA and BrAA showed that the substitution with bromine at position 6 of AA enhances the affinity for the SVCT2 transporter (K_t for AA = 36 ± 3 μM; K_t for BrAA = 5.1 ± 0.4 μM) without impairing the uptake into HRPE cells (V_{max} for AA and BrAA are 4.3 ± 0.3 and 4.0 ± 0.3 nmol/10^6 cells/60 min, respectively).[10]

These interesting data suggest that modifications at position 6 on AA, might be useful to design of a second generation class of compounds, based on BrAA, endowed with increased affinity. Thus, these new conjugates were synthesized as derivatives at position 5 on BrAA of the same model drugs. The interaction with SVCT2 transporters, concerning the parent nipecotic, kynurenic (**2**) and diclofenamic (**3**) acids and their AA and BrAA conjugates was then investigated. As reported in Table 1, the K_i values of AA (20.1 ± 1.6 μM) and BrAA (2.69 ± 0.13), obtained by inhibition experiments, confirm that the 6-Br-substituent on AA enhances the affinity for SVCT2 of about one order of magnitude. Data reported in Table 1 confirm that nipecotic and kynurenic acids are totally unable to interact with SVCT2 transporters, whereas their AA-conjugates show a significant inhibition potency on [^{14}C]AA uptake (Table 1). In view of the fact that chirality of the model drug may also affect transporters interactions, the effects of *R*- and *S*-nipecotic acid on the properties of the respective AA-conjugates has been investigated. As shown in Figure 4, no significant differences have been found among the inhibitory properties of the AA–(*R*,*S*)-Nipec mixture or the AA–(*R*)-Nipec or AA–(*S*)-Nipec diasteroisomers. In fact, their K_i values are in the same range (Table 1). These results show that the SVCT2 transporters interacts with the ascorbic acid conjugate of nipecotic acid irrespectively from its chirality.

On the other hand, as reported in Table 1, we have found a remarkable increase on SVCT2 affinity going from **11** (K_i = 1187 ± 78 μM) to **19** (K_i = 193 ± 14 μM).

A similar behaviour has been found also for the kynurenic acid conjugates with AA (**12**, $K_i = 39.8 \pm 3.2\,\mu M$) and BrAA (**20**, $K_i = 7.4 \pm 0.8\,\mu M$). Taken together, these data are in agreement with our initial hypothesis on the possibility to achieve better affinities by conjugation with BrAA rather than AA, but also consent to suppose the presence of accessory interactions that may improve transporters recognition. Similarly to the AA conjugates of nipecotic and kynurenic acids,[10] the BrAA derivatives interact toward SVCT2 as competitive inhibitors (Fig. 3). Because a competitive inhibition is indicative of an interaction with SVCT2 in the same binding site of AA, this implies that AA- and BrAA-conjugates may be potentially transported by SVCT2, while the parent compounds are certainly not. These findings are of great significance for the design of newer derivatives endowed with improved affinity. However, inhibition experiments performed on HRPE cells using diclofenamic acid (**3**) and its AA or BrAA conjugates **13** and **21**, (Fig. 4), showed a completely different pattern with respect to nipecotic and kynurenic acids (**2**). In fact, as previously anticipated by us,[10] diclofenamic acid (**3**) is able itself to interact with the SVCT2 transporters with an affinity ($K_i = 3.35 \pm 0.16\,\mu M$) similar to that of BrAA. Moreover, upon conjugation with AA (**13**), the inhibition potency of diclofenamic acid becomes two order of magnitude higher ($K_i = 0.19 \pm 0.01\,\mu M$) with respect to the endogenous ligand (AA). These data, completely unexpected, disclosed further perspectives. Therefore, the conjugation of diclofenamic acid with BrAA (**21**) was investigated, resulting in a drastic decrease in the affinity with respect to both the parent drug (**3**) and the AA-conjugate (**13**). In fact, the K_i value of this BrAA-conjugate **21** ($K_i = 21.4 \pm 1.8\,\mu M$) appears similar to that of the endogenous ligand (Table 1).

4. Conclusions

In view of the interesting results previously communicated by us, we have prepared a second generation compounds, based on BrAA, that consented us to confirm our previous hypotheses, further validating our approach. Surprisingly, diclofenamic acid behaved completely different than nipecotic and kynurenic acids, indeed: (i) it was able itself to interact with the transporters but by with a kind of pattern, different from AA and its conjugates; (ii) conjugation to BrAA, differently from conjugation to AA, resulted detrimental for the interaction with the transporters.

Moreover, in the case of *R*- and *S*-nipecotic acid derivatives, the interaction and transport occurred irrespectively of the chirality of the conjugated drug. These data, are in agreement with the inability of nipecotic acid itself to interact with transporters, being only transported after conjugation to AA or BrAA.

5. Experimental

Reaction courses and product mixtures were routinely monitored by thin-layer chromatography (TLC) on silica gel precoated F254 Merck plates with detection under 254 nm UV lamp and/or by spraying with a diluted potassium permanganate solution. Nuclear magnetic resonance (^{1}H NMR) spectra were determined for solution in $CDCl_3$–CD_3OD–DMSO-d_6 on a Bruker AC-200 spectrometer and peak positions are given in parts per million (δ) downfield from tetramethylsilane as internal standard, whereas coupling constants (J) in Hertz. Melting points were obtained in open capillary tubes and are uncorrected. Column chromatography was performed with Merck 60–200 mesh silica gel. Ambient temperature was 22–25 °C. All drying operations were performed over anhydrous magnesium or sodium sulfate. Microanalysis, unless indicated, were in agreement with calculated values within ±0.4%.

5.1. Preparation of 4-hydroxyquinoline-2-carboxylic-acid methyl ester (4)

Kynurenic acid (500 mg, 2.65 mmol) was suspended in distilled MeOH (25 mL) and 96% H_2SO_4 (30–40 drops) was added. The clear solution was heated at reflux for 24 h (TLC, CH_2Cl_2/MeOH, 9/1) and the solvent was evaporated under vacuo, the residue was dissolved in EtOAc and washed with $NaHCO_3$ satd solution. The organic phase was dried and evaporated under vacuo. The crude residue was purified by column chromatography (AcOEt/hexane gradient 1/1 to 8/2). Evaporation of appropriate fractions gave the expected compound **4**.

Pale yellow solid, mp 227,[29] yield 75%. ^{1}H NMR (DMSO-d_6): δ 3.96 (s, 3H, CH_3), 6.66 (s, 1H, C3–H), 7.33–7.41 (m, 1H, C7–H, Ph), 7.65–7.79 (m, 1H, C6–H), 7.94 (d, 1H, $J = 8.32$ Hz, C5–H), 8.08 (d, 1H, $J = 8.17$ Hz, C8–H), 11.9 (br s, 1H, OH Ar). MALDI-TOF MS: m/z 204.5 Da $(M+H)^+$ $C_{11}H_9NO_3$ requires 203.19.

5.2. Preparation of 4-benzyloxyquinoline-2-carboxylic-acid (5)

To a stirred solution, of **4** (180 mg, 0.887 mmol) in anhydrous DMF, anhydrous K_2CO_3 (146.5 mg, 1.06 mmol) was added. Benzyl bromide (126 μL, 1.06 mmol) was then added dropwise, and the resulting mixture was heated at reflux under argon atmosphere. After 4 h, solvent was removed by vacuum. The residue was dissolved in H_2O/MeOH (5.25 mL, 1:1) and NaOH (0.1 mmol) was added and the solution stirred at room temperature. After 2 h, the MeOH was removed and the resulting aqueous solution was acidified (pH 4–5), with 2 M HCl, and then extracted with EtOAc (3×30 mL). The combined organic phases were dried and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (CH_2Cl_2/MeOH, 9/1) to provide 220 mg of **5**.

White solid, mp 143–146 °C. ^{1}H NMR ($CDCl_3$): δ 5.44 (s, 2H, CH_2–Ph), 7.29–7.78 (m, 6H, C3–H and Ph), 7.80–78.05 (m, 2H, C6–H and C7–H), 8.29 (d, 1H, $J = 8.2$ Hz, C5–H), 8.56 (d, 1H, $J = 8.3$ Hz, C8–H). MALDI-TOF MS: m/z 280.2 Da $(M+H)^+$ $C_{17}H_{13}NO_3$ requires 279.29.

5.3. General synthetic procedure for the preparation of the esters 7–9, 14–16

To a stirred solution of the appropriate carboxylic acid derivative (4.36 mmol), DMAP (53 mg, 0.436 mmol) and 3,4-dibenzyloxy-5-(1,2-dihydroxy-ethyl)-5*H*-furan-2-one (1.55 g, 4.36 mmol) in anhydrous CH_2Cl_2 (50 mL) at 0 °C DCC (1.33 mg, 6.49 mmol) was added and the mixture was stirred at room temperature and under argon atmosphere. The reaction was monitored by TLC (EtOAc/hexane, 4/6) and when no further improvement in the formation of the expected product was observed, the mixture was filtered and CH_2Cl_2 was removed in vacuo. The solution was then taken up in EtOAc, washed with 0.5 M HCl (20 mL), brine (20 mL), satd $NaHCO_3$ (20 mL) and dried. The organic layer was dried and removed in vacuo and the crude residue was purified by chromatography eluting with EtOAc/hexane (gradient 1/9 → 4/6) to provide the expected 6-ascorbic acid esters. The formation of the other two possible derivatives, namely the 5-esters and/or 6,5-diesters at the ascorbyl moiety, was also observed, in 5–25% ratio (HPLC), as indicated below. Compound **7** was obtained either as a mixture of two diastereoisomers starting from racemic nipecotic acid, or as the pure *R*,*S*,*R* and *S*,*S*,*R* diasteroisomers starting from *R*- and *S*-nipecotic acid, respectively. These latter were obtained as reported in literature.[30]

5.4. *N*-Boc-piperidine-3-(*R*,*S*/*R*/*S*)-carboxylic acid [2-(3,4-dibenzyloxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-(*S*)-hydroxy-ethyl] ester (7a), related positional isomer (7b) and diester (7c)

Compound **7a** was obtained as a mixture of the two diastereoisomers and/or as the single diastereoisomer (3*R*,2*S*,2*R* and 3*S*,2*S*,2*R*): white foam, yield 60%. ^{1}H NMR ($CDCl_3$): δ 1.30–2.00 (m, 13H, C5–H, C4–H, Boc), 2.44–2.68 (m, 1H, C3–H), 3.2–3.8 (m, 4H, C6–H and C2–H), 3.98–4.3 (m, 3H, hydroxy-ethyl C1–H and C2–H), 4.65 (d, 1H, J = 2 Hz, furan C2–H), 5.05–5.30 (m, 4H, CH_2–Ph), 7.14–7.48 (m, 10H, Ph).

MALDI-TOF MS: *m*/*z* 568.8 Da $(M+H)^+$; 591.1 Da $(M+Na)^+$; 607.2 $(M+K)^+$, $C_{31}H_{37}NO_9$ requires 567.63.

Compound **7a** (3*R*,2*S*,2*R*) obtained from *R*-nipecotic acid: pale yellow foam, yield 30%. ^{1}H NMR ($CDCl_3$) diagnostic proton: 2.43–2.61 (m, 1H, C3–H), 4.64 (d, 1H, J = 1.8 Hz, furan C2–H). MALDI-TOF MS: *m*/*z* 590.8 Da $(M+Na)^+$; 606.1 $(M+K)^+$, $C_{31}H_{37}NO_9$ requires 567.63.

Compound **7a** (3*S*,2*S*,2*R*) obtained from *S*-nipecotic acid: pale yellow foam, yield 45%. ^{1}H NMR ($CDCl_3$) diagnostic proton: 2.46–2.64 (m, 1H, C3–H), 4.645 (d, 1H, J = 2 Hz, furan C2–H). MALDI-TOF MS: *m*/*z* 590.8 Da $(M+Na)^+$; 605.1 $(M+K)^+$, $C_{31}H_{37}NO_9$ requires 567.63.

Compound **7b** (3*R*,1*S*,2*R* and 3*S*,2*S*,2*R*): pale yellow foam, yield 5%. ^{1}H NMR ($CDCl_3$), diagnostic proton: 4.88 (d, 1H, J = 2.2 Hz, furan C2–H). MALDI-TOF MS: *m*/*z* 591.1 Da $(M+Na)^+$; 607.2 $(M+K)^+$, $C_{31}H_{37}NO_9$ requires 567.63.

Compound **7c** (3*R*,2*S*,2*R* and 3*S*,2*S*,2*R* of both positional isomers): pale yellow foam, yield 20%. ^{1}H NMR ($CDCl_3$), δ: diagnostic proton 4.83 (d, 1H, J = 2 Hz, furan C2–H). MALDI-TOF MS: *m*/*z* 779.9 Da $(M+H)^+$; 802.4 Da $(M+Na)^+$; 818.4 $(M+K)^+$, $C_{42}H_{54}N_2O_{12}$ requires 778.37.

5.5. *N*-Boc-piperidine-3-(*R*,*S*)-carboxylic acid [1-(*S*)-(3,4-dibenzyloxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-bromo-ethyl] ester (15)

White foam, yield 60%. ^{1}H NMR ($CDCl_3$): δ 1.40–1.75 (m, 12H, C5–H, C4–H and Boc), 1.90–2.08 (m, 1H, C4–H), 2.25–2.47 (m, 1H, C3–H), 2.60–2.95 (m, 2H, C6–H and C2–H), 3.43–3.58 (m, 2H, hydroxy-ethyl C2–H), 3.82–4.26 (m, 2H, C6–H and C2–H), 5.01 (d, 1H, J = 2.19 Hz, furan C2–H), 5.06–5.22 (m, 4H, CH_2–Ph), 5.28–5.38 (m, 1H, hydroxy-ethyl C1–H), 7.17–7.51 (m, 10H, Ph). MALDI-TOF MS: *m*/*z* 631.1 Da $(M+H)^+$; 654.1 Da $(M+Na)^+$; 670.0 Da $(M+K)^+$, $C_{31}H_{36}BrNO_8$ requires 630.52.

5.6. 4-Benzyloxyquinoline-2-carboxylic acid [2-(3,4-dibenzyloxy-5-oxo-2,5-dihydrofuran-2 (*R*)-yl)-2-(*S*)-hydroxy-ethyl] ester (8a) and related positional isomer (8b)

Compound **8a**: white solid, mp 154–156 °C, yield 56%. ^{1}H NMR ($CDCl_3$): δ 4.39–4.45 (m, 1H, hydroxy-ethyl C2–H), 4.55–4.70 (m, 2H, hydroxy-ethyl C1–H), 4.76 (d, 1H, J = 1.84 Hz, furan C2–H), 5.1–5.29 (m, 6H, CH_2–Ph), 7.10–7.65 (m, 17H, C3–H, C7–H and Ph), 7.68–7.78 (m, 1H, C6–H), 8.14 (d, 1H, J = 8.33 Hz, C5–H), 8.25 (d, 1H, J = 8.49 Hz, C8–H). MALDI-TOF MS: *m*/*z* 618.4 Da $(M+H)^+$; 640.8 Da $(M+Na)^+$; 657.2 Da $(M+K)^+$, $C_{37}H_{31}NO_8$ requires 617.64.

Compound **8b**: pale yellow oil, yield 5%. ^{1}H NMR ($CDCl_3$): diagnostic proton: δ 4.90–4.95 (br, 1H, furan C2–H). MALDI-TOF MS: *m*/*z* 879.6 Da $(M+H)^+$; 901.6 Da $(M+Na)^+$; 917.8 Da $(M+K)^+$, $C_{54}H_{42}N_2O_{10}$ requires 878.92.

5.7. 4-Benzyloxyquinoline-2-carboxylic acid [1-(*S*)-(3,4-dibenzyloxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-bromo-ethyl] ester (16)

White solid, mp 151–154 °C, yield 60%. ^{1}H NMR ($CDCl_3$): δ 3.67–3.85 (m, 2H, hydroxy-ethyl C2–H), 4.94–5.35 (m, 7H, furan C2–H and CH_2–Ph), 5.54–5.63 (m, 1H, hydroxy-ethyl C1–H), 7.05–7.85 (m, 18H, C3–H, C6–H and C7–H, Ph), 8.22–8.35 (m, 2H, C5–H and C8–H).

MALDI-TOF MS: *m*/*z* 681.0 Da $(M+H)^+$; 704.2 Da $(M+Na)^+$; 720.0 Da $(M+K)^+$, $C_{37}H_{30}BrNO_7$ requires 680.54.

5.8. [2-(2,6-Dichlorophenylamino)-phenyl]-acetic acid [2-(3,4-dibenzyloxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-(*S*)-hydroxy-ethyl] ester (9a), related positional isomer (9b) and diester (9c)

Compound **9a**: white foam, yield 65%. ^{1}H NMR ($CDCl_3$): δ 3.84 (s, 2H, CH_2–Ar), 4.0–4.16 (m, 1H, hydroxy-ethyl C2–H), 4.3, 4.4 (ABX, sist. 2H, J_{AB} = 11.54 Hz, J_{AX} = 6.95, J_{BX} = 4.82 Hz hydroxy-ethyl C1–H), 4.64 (d, 1H, J = 2.07 Hz, furan C2–H), 5.06–5.22 (m, 4H, CH_2–Ph), 6.53 (d, 1H, J = 7.93 Hz, phenyl-acetyl H3), 6.90–7.40 (m, 16H, Ph), 6.73 (s, 1H, NH). MALDI-TOF MS: *m/z* 635.4 Da $(M+H)^+$; 658.5 Da $(M+Na)^+$; 674.9 Da $(M+K)^+$, $C_{34}H_{29}Cl_2NO_7$ requires 634.50.

Compound **9b**: pale yellow oil, yield 15%. ^{1}H NMR ($CDCl_3$), δ: diagnostic proton 4.80 (d, 1H, furan C2–H). MALDI-TOF MS: *m/z* 658.4 Da $(M+Na)^+$; 674.5 Da $(M+K)^+$, $C_{34}H_{29}Cl_2NO_7$ requires 634.50.

Compound **9c**: pale yellow oil, yield 15%. ^{1}H NMR ($CDCl_3$), δ: diagnostic proton 4.77 (d, 1H, J = 1.88 Hz, furan C2–H). MALDI-TOF MS: *m/z* 936.0 Da $(M+Na)^+$; 952.2 $(M+K)^+$, $C_{48}H_{38}Cl_4N_2O_8$ requires 912.63.

5.9. [2-(2,6-Dichlorophenylamino)-phenyl]-acetic acid [1-(*S*)-(3,4-dibenzyloxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-bromo-ethyl] ester (17)

Yellow foam, yield 45%. ^{1}H NMR ($CDCl_3$): δ 3.50–3.59 (m, 2H, hydroxy-ethyl C2–H), 3.62–3.90 (m, 2H, CH_2–Ar), 4.80–5.12 (m, 5H, furan C2–H and 4H, CH_2–Ph), 5.30–5.40 (m, 1H, hydroxy-ethyl C1–H), 6.53 (d, 1H, J = 7.92 Hz, phenyl-acetyl H3), 6.82–7.59 (m, 17H, Ph and NH). MALDI-TOF MS: *m/z* 698.2 Da $(M+H)^+$; 720.9 Da $(M+Na)^+$; 737.1 Da $(M+K)^+$, $C_{34}H_{28}BrCl_2NO_6$ requires 697.40.

5.10. Piperidine-3(*R*,*S*/*R*/*S*)-carboxylic acid [2-(3,4-dibenzyloxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-(*S*)-hydroxy-ethyl] ester (10)

To a stirred solution, of **7a** (1.21 g, 2.1 mmol) in anhydrous CH_2Cl_2 was added, in a dropwise manner, TFA (3.8 mL) and the mixture was stirred for 2 h under argon atmosphere. Most of solvent was then removed in vacuo and the residue was added with CH_2Cl_2 (20 mL) and the solution washed with $NaHCO_3$ satd (2×20 mL). The organic layer was dried and the solvent removed in vacuo. The crude residue was purified by column chromatography (CH_2Cl_2/MeOH 9/1) to provide 630 mg of **10**.

Pale yellow oil, yield 63%. ^{1}H NMR ($CDCl_3$): δ 1.63–2.10 (m, 4H, C5–H, C4–H), 2.65–3.50 (m, 5H, C3–H, C6–H, C2–H), 4.05–4.59 (m, 3H, hydroxy-ethyl C2–H and C1–H), 4.64 (d, 1H, J = 2 Hz, furan C2–H), 4.90–6.00 (m, 4H, CH_2–Ph and NH), 7.05–7.5 (m, 10H, Ph). MALDI-TOF MS: *m/z* 469.0 Da $(M+H)^+$; 490.7 $(M+Na)^+$; 506.6 $(M+K)^+$, $C_{26}H_{29}NO_7$ requires 467.51.

Compound **10** (3*R*,2*S*,2*R*) obtained from *R*-nipecotic acid: pale yellow foam, yield 54%. ^{1}H NMR ($CDCl_3$) diagnostic proton: 4.64 (d, 1H, J = 2 Hz, furan C2–H). MALDI-TOF MS: *m/z* 467.6 Da $(M+H)^+$; 489.8 Da $(M+Na)^+$; 505.9 $(M+K)^+$, $C_{26}H_{29}NO_7$ requires 467.51.

Compound **10** (3*S*,2*S*,2*R*) obtained from *S*-nipecotic acid: pale yellow foam, yield 48%. ^{1}H NMR ($CDCl_3$) diagnostic proton: 4.64 (d, 1H, J = 2 Hz, furan C2–H). MALDI-TOF MS: *m/z* 467.6 Da $(M+H)^+$; 489.8 Da $(M+Na)^+$; 505.9 $(M+K)^+$, $C_{26}H_{29}NO_7$ requires 467.51.

5.11. Piperidine-3-(*R*,*S*)-carboxylic acid [1-(*S*)-(3,4-dibenzyloxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-bromo-ethyl] ester (18)

Yellow oil, yield 70%. ^{1}H NMR ($CDCl_3$): δ 1.40–1.76 (m, 3H, C5–H, C4–H), 1.81–2.17 (m, 1H, C4–H), 2.60–3.20 (m, 5H, C3–H, C6–H and C2–H), 3.45–3.61 (m, 2H, hydroxy-ethyl C2–H), 5.01 (d, 1H, J = 1.78 Hz, furan C2–H), 5.05–5.25 (m, 4H, CH_2–Ph), 5.28–5.38 (m, 1H, hydroxy-ethyl C1–H), 7.20–7.50 (m, 10H, Ph). MALDI-TOF MS: *m/z* 531.9 Da $(M+H)^+$; 553.6 Da $(M+Na)^+$; 569.7 Da $(M+K)^+$, $C_{26}H_{28}BrNO_6$ requires 530.41.

5.12. General procedure to remove the benzyl protecting groups

To a solution of the appropriate esters **8a**, **9a**, **10**, **16**–**18** (2.14 mmol) in MeOH or EtOAc (50 mL) 10% Pd/C (200 mg) was added, and the mixture was stirred 5–24 h under hydrogen atmosphere. After complete disappearance of the starting material (TLC, CH_2Cl_2/MeOH, 9/1) the mixture was filtered on Celite pad and the solvent was removed in vacuo. The crude residue was purified by column chromatography (CH_2Cl_2/MeOH gradient from 9/1 to 7/3) to give the expected deprotected compounds **11**–**13**, **19**–**21**.

5.13. Piperidine-3-(*R*,*S*/*R*/*S*)-carboxylic acid [2-(3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-(*S*)-hydroxy-ethyl] ester (11)

Compound **11** was obtained as a mixture of the two diastereoisomers and/or as the single diasteroisomer. Mixture: pale yellow foam, yield 30%. ^{1}H NMR (DMSO-d_6): δ 1.40–1.74 (m, 3H, C5–H), 1.76–1.94 (m, 1H, C4–H), 2.57–3.20 (m, 5H, C3–H, C6–H and C2–H), 3.78–4.35 (m, 4H, hydroxy-ethyl C1–H, C2–H and furan C2–H), 6.17 (br s, 1H, NH). MALDI-TOF MS: *m/z* 288.7 Da $(M+H)^+$, $C_{12}H_{17}NO_7$ requires 287.27. Anal. ($C_{12}H_{17}NO_7$) C, H, N.

Compound **11** (3*R*,2*S*,2*R*) obtained from *R*-nipecotic acid: pale yellow solid, mp 58–60 °C, yield 24%. $[\alpha]_D^{25}$ + 33 (*c* 0.41%, H_2O); ^{1}H NMR (DMSO-d_6) diagnostic proton: 2.30–2.38 (m, 1H, C3–H), 4.31 (d, 1H, J = 3 Hz, furan C2–H). MALDI-TOF MS: *m/z* 288.8 Da $(M+H)^+$; 309.8 Da $(M+Na)^+$; 325.7 $(M+K)^+$, $C_{12}H_{17}NO_7$ requires 287.27. Anal. ($C_{12}H_{17}NO_7$) C, H, N.

Compound **11** (3*S*,2*S*,2*R*) obtained from *S*-nipecotic acid: pale yellow solid, mp 60–62 °C, yield 39%. $[\alpha]_D^{25}$ +42 (*c* 0.4%, H_2O); ^1H NMR (DMSO-d_6) diagnostic proton: 2.58–2.72 (m, 1H, C3–H), 4.26 (d, 1H, *J* = 2.8 Hz, furan C2–H). MALDI-TOF MS: *m*/*z* 288.0 Da $(M+H)^+$, 309.9 Da $(M+Na)^+$; 325.7 $(M+K)^+$, $C_{12}H_{17}NO_7$ requires 287.27. Anal. ($C_{12}H_{17}NO_7$) C, H, N.

5.14. Piperidine-3-(*R*,*S*)-carboxylic acid [1-(*S*)-(3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-bromo-ethyl] ester (19)

Yellow solid, mp 140–143 °C, yield 30%. ^1H NMR (CD_3OD-d_6): δ 1.67–2.13 (m, 4H, C5–H, C4–H), 2.8–3.88 (m, 7H, C3–H, C6–H, C2–H and hydroxy-ethyl C2–H), 4.64 (d, 1H, *J* = 2.13 Hz, furan C2–H), 5.48–5.58 (m, 1H, hydroxy-ethyl C1–H). MALDI-TOF MS: *m*/*z* 350.7–352.5 Da $(M+H)^+$; 391.0–392.6 Da $(M+K)^+$, $C1_2H_{16}BrNO_6$ requires 350.16. Anal. ($C_{12}H_{16}BrNO_6$) C, H, N: C calcd 41.16; found 40.91.

5.15. Hydroxyquinoline-2-carboxylic acid [2-(3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-(*S*)-hydroxy-ethyl] ester (12)

White solid, mp 138 °C, yield 40%. ^1H NMR (DMSO-d_6): δ 4.12–4.28 (m, 1H, hydroxy-ethyl C1–H), 4.40–4.52 (m, 2H, hydroxy-ethyl C2–H), 4.87 (d, 1H, *J* = 1.8 Hz, furan C2–H), 6.78 (s, 1H, C3–H), 7.30–7.47 (m, 1H, C7–H), 7.65–7.80 (m, 1H, C6–H), 7.95 (d, 1H, *J* = 8.4 Hz, C5–H), 8.08 (d, 1H, *J* = 8.8 Hz, C8–H), 8.46 (br s, 1H, furan C4–OH), 11.24 (br s, 1H, furan C3–OH), 12.06 (br s, 1H, OH Ar). MALDI-TOF MS: *m*/*z* 348.5 Da $(M+H)^+$; 370.8 Da $(M+Na)^+$; 386.8 Da $(M+K)^+$, $C_{16}H_{13}NO_8$ requires 347.28. Anal. ($C_{16}H_{13}NO_8$) C, H, N: H calcd 3.77; found 3.75.

5.16. Hydroxyquinoline-2-carboxylic acid [1-(*S*)-(3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-bromo-ethyl] ester (20)

White solid, mp 203–206 °C, yield 74%. ^1H NMR (DMSO-d_6): δ 3.81–4.05 (m, 2H, hydroxy-ethyl C2–H), 5.15 (d, 1H, *J* = 2.13 Hz, furan C2–H), 5.63–5.71 (m, 1H, hydroxy-ethyl C1–H), 6.56 (s, 1H, C3–H), 7.28–7.44 (m, 1H, C7–H), 7.65–7.80 (m, 1H, C6–H), 7.93 (d, 1H, *J* = 8.26 Hz, C5–H), 8.08 (d, 1H, *J* = 7.7 Hz, C8–H), 12.12 (br s, 1H, OHAr). MALDI-TOF MS: *m*/*z* 411.9 Da $(M+H)^+$; 432.8 Da $(M+Na)^+$; 449.3 Da $(M+K)^+$, $C_{16}H_{12}BrNO_7$ requires 410.17. Anal. ($C_{16}H_{12}BrNO_7$) C, H, N.

5.17. [2-(2,6-Dichlorophenylamino)-phenyl]-acetic acid [2-(3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-(*R*)-yl)-2-(*S*)-hydroxy-ethyl] ester (13)

White solid, mp 152–154 °C, yield 73%. ^1H NMR (DMSO-d_6): δ 3.84 (s, 2H, CH_2–Ar), 3.93–4.14 (m, 3H, hydroxy-ethyl C1–H and C2–H), 4.59 (d, 1H, *J* = 2.41 Hz, furan C2–H), 6.23 (d, 1H, *J* = 7.93 Hz, phenyl-acetyl H3), 6.79–6.90 (m, 1H, *J* = 6.98 Hz, phenyl-acetyl H3), 7.01–7.30 (m, 4H, Ph and NH), 7.53 (d, 2H, *J* = 8.02 Hz, phenyl-amino H3 and H5). MALDI-TOF MS: *m*/*z* 477.6 Da $(M+Na)^+$; 493.9 Da $(M+K)^+$, $C_{20}H_{17}Cl_2NO_7$ requires 454.26. Anal. ($C_{20}H_{17}Cl_2NO_7$) C, H, N.

5.18. [2-(2,6-Dichlorophenylamino)-phenyl]-acetic acid [1-(*S*)-(3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-yl)-2-bromo-ethyl] ester (21)

White foam, yield 35%. ^1H NMR (DMSO-d_6): δ 3.58–3.98 (m, 4H, CH_2–Ar and hydroxy-ethyl C2–H), 5.09 (d, 1H, *J* = 2.16 Hz, furan C2–H), 5.39–5.52 (m, 1H, hydroxy-ethyl C1–H), 6.25 (d, 1H, *J* = 7.08 Hz, phenyl-acetyl H3), 6.80–6.98 (m, 2H, phenyl-acetyl H5 and NH), 7.02–7.30 (m, 3H, phenyl-acetyl H4/H6 and phenyl-amino H4), 7.52 (d, 2H, *J* = 7.99 Hz, phenyl-amino H3, H5), 8.72 (br s, 1H, furan C4–OH), 11.45 (br s, 1H, furan C3-OH).

MALDI-TOF MS: *m*/*z* 518.2 Da $(M+H)^+$; 541.0 Da $(M+Na)^+$; 557.0 Da $(M+K)^+$, $C_{20}H_{16}BrCl_2NO_6$ requires 517.15. Anal. ($C_{20}H_{16}BrCl_2NO_6$) C, H, N.

5.19. Stability

The stability in water (pH 7 and 1.2) and at enzymatic hydrolysis (porcine liver esterase, PLE) of test compounds was assayed as described previously.[28] Compounds were stable in water from 24 h to 4 weeks and showed halflives ranging from 60 to >180 min (**10** > **6** > **4**) in the case of PLE enzymatic hydrolysis.

5.20. HRPE cell culture

HRPE cell line was routinely grown in 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media, supplemented with 10% FBS, 50 μmg/mL streptomycin and 50 IU/mL penicillin (Gibco Laboratories, Invitrogen Italia, Milan, Italy) at 37 °C in 5% CO_2. Cells employed for uptake measurements[10] were seeded in 24-well tissue culture plates and grown to confluence (2–3 days).

5.21. SVCT2 transporter interactions

Transport assays were performed following the method described by Rajan et al.[21] Briefly, the uptake buffer was prepared fresh each time, the composition was: 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose. DTT (1 mM) (Sigma, St. Louis, MO, USA) was also added to the uptake buffer to prevent the oxidation of AA. At this concentration, DTT had no effect on the transport process. Incubation time for the transport measurements was 60 min (within the time course of linear uptake, Fig. 2) at 37 °C, after this time the uptake buffer containing the radioactive substrate was aspirated off and cells were washed with 2 × 2 mL of ice-cold uptake buffer. Cells were then solubilized in 250 μL of 0.2 M NaOH solution containing 0.5% CHAPS (Sigma), transferred to vials and radioactivity associated with the cells was evaluated by liquid scintillation spectrometry.

The kinetics of [^{14}C]AA (6 mCi/mmol; NEN Life Science, Boston, MA, USA) uptake, mediated by SVCT2, was analyzed using concentration of AA ranging from 2.5 to 600 μM. The concentration of [^{14}C]AA ranged from 2.5 to 50 μM and was kept constant at 50 μM. Data were analyzed by nonlinear regression of Michaelis–Menten equation.

Inhibition of AA transport was determined by adding the indicated concentrations of unlabelled compounds to plated cells along with either [^{14}C]AA at fixed concentration 50 μM, or [^{14}C]AA ranging from 2.5 to 100 μM. The unlabelled inhibitor concentrations displacing 50% of [^{14}C]AA (IC_{50} values) were obtained by computer analysis of displacement curves. Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1+[C^*]/K_t^*)$, where $[C^*]$ is the concentration of the [^{14}C]AA and K_t^* its Michaelis–Menten constant.[29] K_i values were also obtained by Lineweaver–Burk analysis for competitive inhibitors, using the equation $K_i = [\text{inhibitor}]/\{(K_{M,obs}/K_M)-1.0\}$, where $K_{M,obs}$ is the apparent Michaelis–Menten constant obtained in the presence of a fixed concentration of inhibitor. All calculations were performed using the computer program Graph Pad Prism (GRAPHPAD).

Statistical analysis was performed by ANOVA followed by Dunnett's *t*-test. Difference was considered statistically significant at *P* values less than 0.05.

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Appendix A. Elemental analyses

Compound	Formula	Anal. Calcd (found)		
		H	C	N
11	$C_{12}H_{17}NO_7$	5.96 (5.94)	50.17 (50.19)	4.88 (4.87)
11 (3*R*,2*R*,1*S*)	$C_{12}H_{17}NO_7$	5.96 (5.95)	50.17 (50.20)	4.88 (4.88)
11 (3*S*,2*R*,1*S*)	$C_{12}H_{17}NO_7$	5.96 (5.94)	50.17 (50.17)	4.88 (4.87)
12	$C_{16}H_{13}NO_8$	3.77 (3.75)	55.34 (55.30)	4.03 (4.02)
13	$C_{20}H_{17}Cl_2NO_7$	3.77 (3.77)	55.88 (55.91)	3.08 (3.09)
19	$C_{12}H_{16}BrNO_6$	4.61 (4.60)	41.16 (40.91)	4.00 (4.01)
20	$C_{16}H_{12}BrNO_7$	2.95 (2.94)	46.85 (46.88)	3.41 (3.40)
21	$C_{20}H_{16}BrCl_2NO_6$	3.12 (3.12)	46.46 (46.32)	2.71 (2.72)

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