Letters

Design, Synthesis and Activity of Ascorbic Acid Prodrugs of Nipecotic, Kynurenic and Diclophenamic Acids, Liable to Increase Neurotropic Activity

Stefano Manfredini,*,† Barbara Pavan,‡ Silvia Vertuani,† Martina Scaglianti,† Donatello Compagnone,† Carla Biondi,‡ Angelo Scatturin,† Sergio Tanganelli,§ Luca Ferraro,§ Puttur Prasad,|| and Alessandro Dalpiaz†

Department of Pharmaceutical Sciences, via Fossato di Mortara 19, 44100 Ferrara, Italy, Department of Biology, General Physiology Section, via Borsari 46, 44100 Ferrara, Italy, and Department of Experimental Clinical Medicine, Pharmacology Section, via Fossato di Mortara 19, 44100 Ferrara, Italy, and Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta, GA

Received July 26, 2001

Abstract: To improve the entry of certain drugs into brain, ascorbic acid (AA) conjugates of these drugs were synthesized and their capacity to interact with SVCT2 ascorbate transporters was explored. Kinetic studies clearly indicate that all of the conjugates were able to competitively inhibit ascorbate transport in human retinal pigment epithelial cells (HRPE). In vivo studies, in a mouse model system, demonstrate that conjugate **3** is better absorbed compared to the nonconjugated parent drug.

Introduction. Distribution of drugs to the central nervous system (CNS) is one of the major problems of current therapy of brain diseases. Difficulties in the crossing of the brain barrier often impair efficacy of valuable drugs. In past years, most of the attempts to overcome this drawback have been directed to the amelioration of the lipophilic properties through the preparation of prodrugs by formation of reversible linkage with suitable groups.[1,2] A new approach that takes into account the recent progress of molecular cloning and expression of transporters genes is being applied. It is now clear that membrane transporters of native compounds also take part in drug transport in various tissues. Several specific transporters have been identified, in the brain capillary endothelia, and among them some of those involved in the active supply of nutrients (i.e., glucose, amino acids) have been used to prepare prodrugs with improved CNS penetration.[3] Very recently Na^+-dependent ascorbate transporters, SVCT1 and SVCT2, have been characterized;[4] in particular, expression of SVCT2 is found in the choroid plexus. It has been demonstrated that the highest ascorbate (AA) concentrations in mammalian bodies can be found in the brain, spinal cord, and adrenal glands.[5] Several ways have been proposed for the AA entry into cerebrospinal fluids (CRF),[6,7] and among them the sodium-dependent AA transporters, SVCT2.[8,9] The human SVCT2 isoform has been cloned and characterized by Rajan et al.[10] It can be thus hypothesized that drugs able to interact with the SVCT2 transporters isoform could potentially be transported into the brain.

Taking these premises into account, we started the present study with the aim to investigate conjugation to AA as a possible mean to improve the entry of such CNS drugs that are not effectively delivered to the brain. Thus, we have designed, prepared, and tested model AA conjugates and derivatives to verify our initial hypothesis.

Chemistry. The strategy for the conjugation of AA to the corresponding counterparts was devised considering functional groups not involved with the mechanism of the transport[11] and the formation of a reversible bond to warrant the release of the conjugated drug into the cells. Thus, we have evaluated esters at 6 and 5 positions on AA, and drugs carrying carboxylic acid functions, namely nipecotic acid, kynurenic acid, and diclofenamic acid, were selected as lead compounds. Nipecotic and kynurenic acids needed previous protection at the amino and hydroxyl functions, respectively. Thus nipecotic acid was protected as *tert*-butoxycarbonyl derivative (BOC) as reported in the literature[12] and kynurenic acid as benzyl derivative (**8**). This required prior conversion to the kynurenic acid methyl ester **7**[13] simply accomplished in MeOH under acidic catalysis rather than dry HCl as described previously. Esterification on AA was carried out by a general procedure involving a protected precursor, namely the 2,3-dibenzyl derivative **1**,[14] in the presence of *N*,*N*′-dicyclohexylcarbodimide (DCC) (Schemes 1−3). Mixtures of 6- and/or 5- and/or 5,6-di-*O*-ascorbates were sometimes obtained (**2a**−**c** and **5a**,**c**) with large predominance of the 6-isomers over the other products. The 6-*O*-ascorbates (**2a**, **5a**, **9**) were then deprotected by hydrogenolysis (**3** and **9**) treatment with BCl_3 (**5**) or trifluoro acetic acid (TFA) (**2a**).

The final compounds (**4**, **6**, **10**) were obtained in satisfactory yields (82−40%). The stability in water (pH 7 and pH 1.2) and at enzymatic hydrolysis (porcine liver esterase, PLE) of test compounds was assayed as described previously.[15]

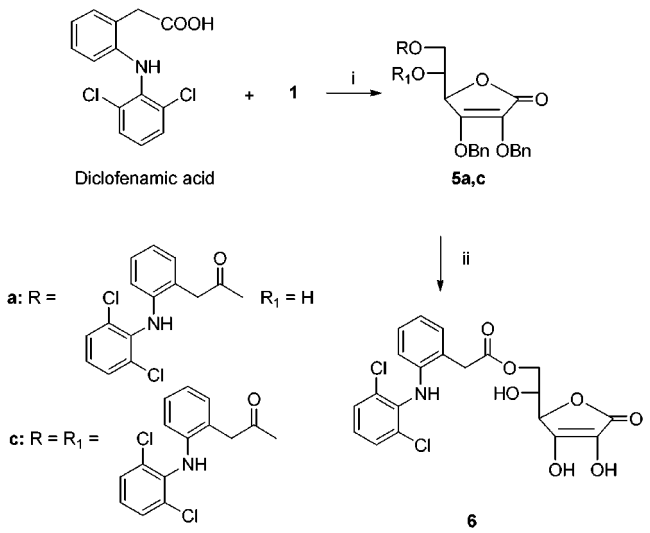
Compounds were stable in water from 24 h to 4 weeks. Stability in PLE enzymatic hydrolysis conditions showed half-lives ranging from 60 min to >180 min (**10** > **6** > **4**).

Biology. The isoform identity of the AA transporters expressed in HRPE cells was determined by RT-PCR analysis. Poly(A)$^+$ RNA isolated from HRPE cells was reverse transcribed using primer pairs specific for SVCT1 and SVCT2. The specificity of the product formed was confirmed by nucleotide sequencing of the

† Department of Pharmaceutical Sciences , Universita degli Studi di Ferrara.
‡ Department of Biology, Universita degli Studi di Ferrara.
§ Department of Experimental Clinical Medicine, Universita degli Studi di Ferrara.
|| Medical College of Georgia.

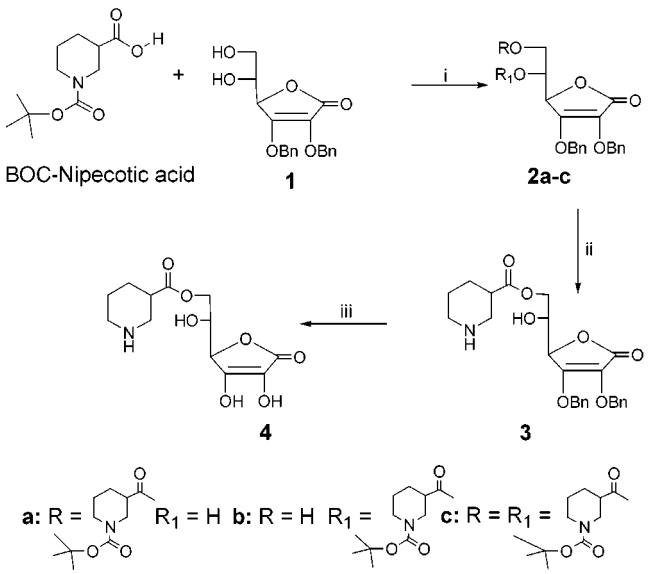
10.1021/jm015556r CCC: $22.00
Published on Web 12/21/2001

Scheme 1[a]



[a] Reagents and conditions: (i) CH_3CN, DMAP, DCC; (ii) TFA, CH_2Cl_2; (iii) CH_3OH, H_2, Pd/C 10%.

Scheme 2[a]



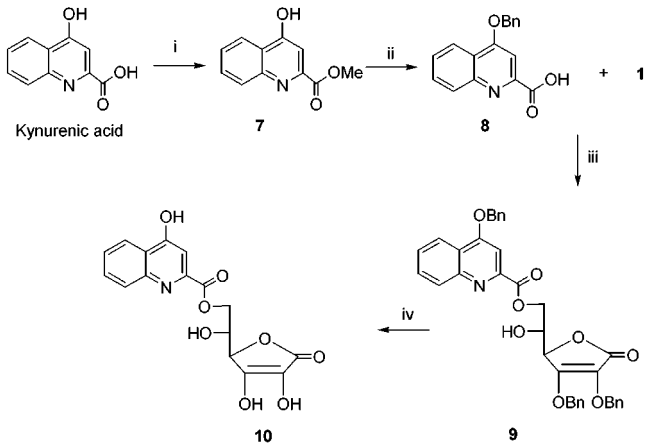
[a] Reagents and conditions: (i) CH_2Cl_2, DMAP, DCC; (ii) CH_2Cl_2, BCl_3, −78 °C.

PCR product. Transport assays were performed in HRPE cells[10] using [^{14}C]AA, and inhibition of AA transport was determined by adding the indicated concentrations of unlabeled compounds to plated cells along either with [^{14}C]AA at a fixed concentration of 50 μM or with [^{14}C]AA ranging from 2.5 to 100 μM. Intracellular accumulation of 6-bromoascorbate (BrAA) was measured by incubating HRPE cells with increasing concentrations of unlabeled analogue, followed by HPLC analysis.

Pharmacology. In a final set of experiments, the nipecotic acid conjugate (**4**), systemically administered, was tested against pentylentetrazol (PTZ)-induced convulsions in mice.

Results and Discussion. Recent cloning studies have shown that two subtypes of AA transporters are present in the mammalian system, namely SVCT1 and SVCT2. These two subtypes share significant homology

Scheme 3[a]



[a] Reagents and conditions: (i) CH_3OH, H_2SO_4 96%, reflux; (ii) (a) benzylbromide, K_2CO_3, DMF; (b) H_2O−MeOH, NaOH; (iii) DMF/CH_2Cl_2, DMAP, DCC; (iv) AcOEt, H_2, Pd/C 10%.

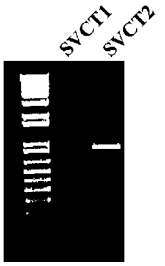


Figure 1. Isoform identity of AA transporter expressed in HRPE cells. Poly(A)$^+$ RNA from HRPE cells was subjected to RT-PCR using primer pairs specific for SVCT1 and SVCT2.

in amino acid sequence and are capable of mediating the Na^+-dependent transport of AA. Previous studies have suggested the presence of SVCT2 in HRPE cells.[10] The isoform identity of the AA transporter expressed in HRPE cells was confirmed by RT-PCR analysis. Poly-(A)$^+$ RNA isolated from HRPE cells was used for RT-PCR with the primer pairs specific for SVCT1 and SVCT2.

Figure 1 describes the results of RT-PCR for the molecular identity of the AA transporter subtype that is expressed in HRPE cells. RT-PCR product of the expected size was obtained only with primers specific for SVCT2 but not for SVCT1. The PCR product obtained was further purified and sequenced to confirm the identity of the amplified product by dideoxy sequencing. This clearly demonstrates that HRPE cells express SVCT2, but not SVCT1. We have therefore chosen HRPE cells as a model for the in vitro analysis of the endogenous activity of SVCT2 transporters. Such a model appears remarkably suitable to study the in vivo transport across the blood−brain barrier (BBB) because RPE and leptomeninges cells are ontogenically and functionally analogues to each other, forming the blood−retina and blood−brain barriers with tight junctions between cells to seal closed compartments of retina and brain.[16]

The uptake of 50 μM [^{14}C]AA was linear for at least 100 min ($r = 0.995$), as determined by initial time-course studies (data not shown). Future experiments were therefore done with 60 min incubation. The kinetic of the [^{14}C]AA uptake mediated by SVCT2 showed rates

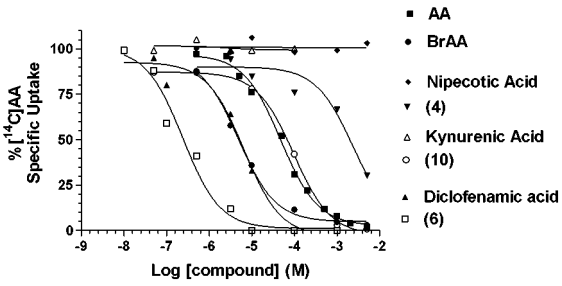


Figure 2. Inhibition of 50 μM [^{14}C]AA uptake by AA, BrAA, kynurenic acid, nipecotic acid, diclofenamic acid, and their AA conjugates (**4**, **6**, and **10**, respectively).

hyperbolically related to AA concentration (2.5−1000 μM), indicating saturability of the uptake process. The related Eadie−Hofstee plot was linear ($n = 8$, $r = 0.991$, $p < 0.0001$), and analogously, computer analysis of the saturation experiment suggested a one-site, rather than two-sites model. The Michaelis−Menten constant (K_t) and V_{max} values for the transport process are 36 ± 3 μM and 4.3 ± 0.3 nmol/10^6 cells/60 min, respectively.

To verify if unlabeled BrAA can be accumulated into HRPE cells by mediated SVCT2 uptake, the HPLC analysis was performed. The rate was found to be hyperbolically related to BrAA concentration (0.5−100 μM), indicating saturability of the uptake process. The related Eadie−Hofstee plot was linear ($n = 8$, $r = 0.994$, $p < 0.0001$), and analogously, computer analysis of the saturation experiment suggested a one-site, rather than two-sites model. The Michaelis−Menten constant (K_t) and V_{max} values for the transport process are 5.1 ± 0.4 μM and 4.0 ± 0.3 nmol/10^6 cells/60 min, respectively.

We have therefore demonstrated, for the first time, that BrAA is actively transported into HRPE cells, interacting with the SVCT2 transporters with an order of magnitude higher than AA; this is indicative that modifications at position 6 on AA are compatible for the design of analogues that may still be able to interact with the transporters.

The uptake rate of 50 μM [^{14}C]AA in the presence of varying concentrations of AA, BrAA, nipecotic, diclofenamic, and kynurenic acids and their conjugates with AA (**4**, **6**, **10**, respectively) are represented in Figure 2.

It can be observed that nipecotic acid and kynurenic acid were not able to inhibit the [^{14}C]AA in the concentration range investigated (0.5−5000 μM). On the other hand, the related conjugates **4**, **10**, AA, and BrAA were all able to inhibit the [^{14}C]AA uptake in a concentration-dependent manner. Among them, BrAA was the most potent whereas **10** and AA showed an intermediate potency. However, of particular relevance, diclofenamic acid and its conjugate, **6**, showed a high inhibition potency. In fact, compound **6**, appears the best inhibitor among all the compounds analyzed. According to the inhibition constant values (K_i), obtained by nonlinear regression analysis of the curves in Figure 2, the order of inhibition potency was as follows: nipecotic and kynurenic acids (no inhibition), compound **4** (K_i = 1033 ± 72 μM), compound **10** (K_i = 40.2 ± 3.4 μM), AA (K_i = 20.1 ± 1.6 μM), BrAA (K_i = 2.69 ± 0.13 μM), diclofenamic acid (K_i = 2.72 ± 0.16 μM), and compound **6** (K_i = 0.16 ± 0.01 μM). On the basis of these data, we next examined inhibition kinetics of the AA analogues in greater detail.

Table 1. K_t and V_{max} Values Obtained by [^{14}C]AA Kinetic Experiments Performed in the Absence and in the Presence of Increasing Concentrations of Inhibitors

compounds	K_t (μM)	V_{max} (nmol/10^6 cells /60 min)
[^{14}C]AA	36 ± 3	4.3 ± 0.3
[^{14}C]AA + (4) (2 mM)	94 ± 4	4.2 ± 0.3
[^{14}C]AA + (4) (5 mM)	165 ± 9[a]	4.1 ± 0.3
[^{14}C]AA + Br AA (75 μM)	816 ± 54[a]	4.1 ± 0.4
[^{14}C]AA + diclofenamic acid (6 μM)	32 ± 3	3.1 ± 0.2[a]
[^{14}C]AA + diclofenamic acid (12 μM)	29 ± 3	2.0 ± 0.2[a]
[^{14}C]AA + diclofenamic acid (24 μM)	31 ± 3	1.4 ± 0.2[a]
[^{14}C]AA + diclofenamic acid (60 μM)	29 ± 3	0.49 ± 0.14[a]
[^{14}C]AA + diclofenamic acid (120 μM)	28 ± 3	0.26 ± 0.11[a]
[^{14}C]AA + (6) (250 nM)	73 ± 5	4.5 ± 0.3
[^{14}C]AA + (6) (750 nM)	188 ± 15[a]	4.1 ± 0.3
[^{14}C]AA + (6) (3 μM)	609 ± 40[a]	4.4 ± 0.3
[^{14}C]AA + (10) (100 μM)	119 ± 6[b]	4.6 ± 0.4
[^{14}C]AA+ (10) (500 μM)	296 ± 21[a]	4.0 ± 0.2

[a] $p < 0.001$, significant versus [^{14}C] ascorbate alone. [b] $p < 0.05$, significant versus [^{14}C] ascorbate alone.

Table 1 reports the K_t and V_{Max} values obtained by kinetic experiments of [^{14}C]AA uptake performed in the absence and in the presence of different concentrations of inhibitors. We can observe that in the presence of increasing concentrations of compounds **4**, BrAA, **6**, and **10**, the K_t values observed increase, whereas the corresponding V_{max} values do not appear affected by the presence of inhibitors. On the other hand, in the presence of increasing concentrations of diclofenamic acid, the K_t values observed do not appear affected by the presence of inhibitor, whereas the V_{max} values drastically decrease. These data suggest that the AA analogues BrAA, **4**, **6**, and **10** competitively inhibit [^{14}C]-AA uptake. The mean of K_i values calculated according to Lineweaver−Burk analysis were as follows: 3.4 ± 0.2 μM for BrAA, 1164 ± 99 μM for compound **4**, 0.20 ± 0.02 μM for compound **6**, and 52.1± 4.6 μM for compound **10**. On the other hand, data from Table 1 suggest that diclofenamic acid inhibits [^{14}C]AA uptake in a noncompetitive manner.

Nipecotic, kynurenic, and diclofenamic acids were selected as model compounds that provided important activity at the CNS level but were endowed with reduced BBB penetration. In these regards:

(i) Nipecotic acid is one of the most potent inhibitors of γ-butyric acid (GABA), with potential applications in CNS disorders such as Parkinson and epilepsy, but which is inactive as an anticonvulsant when administered systemically.[17]

(ii) Kynurenic acid and several excitatory glutamate analogues are valuable molecules in the control of neurodegenerative disorders,[18] but they suffer from restricted brain passage.[19]

(iii) COX-inhibitors, such as diclofenamic acid, are actively studied for their application in Alzheimer's disease (AD)[20] and other important CNS pathologies[21,22] because of their effects on cycloxygenase inhibition.

Taking this into account, we have demonstrated that the parent nipecotic, kynurenic, and diclofenamic acids cannot be transported by the SVCT2 transporter, and that the latter compound interacts in a noncompetitive mechanism. This unprecedented observation implies that an involvement of this mechanism in the side effects of diclofenamic acid and its analogues may be

Table 2. Effect of ip Injection of Nipecotic Acid (0.75 mmol/kg) or 6-*O*-Nipecotil-ascorbate (**4**, 0.75 mmol/kg) on Pentylenetetrazole-Induced Convulsions in Mice

treatment	latency to convulsion (s)
control (saline + PTZ)	621 ± 28
nipecotic acid + PTZ	603 ± 53
4 + PTZ	922 ± 124[a]

[a] $p < 0.05$, significantly different from the other groups, according to ANOVA followed by the Newman−Keuls test for multiple comparisons.

also possible, in view of a consistent reduction of SVCT2 transporters available for the AA transport. However, this consideration requires further investigations.

When nipecotic, kynurenic, and diclofenamic acids were conjugated to AA, all enhanced their ability to interact with SVCT2 transporters in a competitive manner; this implies a potential use of this approach to transport purposes.

In this context, it is interesting to note that diclofenamic acid is converted from a noncompetitive inhibitor to a competitive inhibitor upon conjugation. Further work is in progress to understand this intriguing phenomenon.

To support the possibility that conjugation to AA could be a mean to improve the entry into the brain of drugs that do not easily penetrate the BBB, we evaluated the effects of the systemic injection of the nipecotic acid conjugate (**4**) on PTZ-induced convulsions in mice. Compound **4** was selected in view of the possibility to examine its behavior in a well-known animal model and moreover for its better solubility in water as compared to compound **10**. In the saline group, the sc injection of PTZ (80 mg/kg) induced tonic convulsions with a latency of 621 ± 27 s. As shown in Table 2, the ip injection of **4** (0.75 mmol/kg) significantly increased the latency to appearance of PTZ-induced tonic convulsions, while as expected,[23] nipecotic acid (0.75 mmol/kg) was ineffective. In all groups, no lethality was observed.

The compound **4** and nipecotic acid-treated mice showed no apparent abnormality in general behavior except for a mild diarrhea, which appeared 40 min after the injection only in animals treated with compound **4**.

We can conclude that the conjugation of drugs with AA is an important tool in studying the mechanism of the interaction of AA to its transporters and can open new interesting perspectives for the possibility of obtaining prodrugs effective for the crossing of the BBB by means of endogenous transporters. Further studies are currently ongoing in order to assess and extend this possibility.

Acknowledgment. This work was supported by University of Ferrara (S.M., A.D., A.S., C.B., and S.V.) and National Institutes of Health Grant (HD37150, P.P.).

References

(1) Hansch, C.; Bjorkroth, J. P.; Leo, A. Hydrophobicity and central nervous system agents: on the principle of minimal hydrophobicity in drug design. *J. Pharm. Sci.* **1987**, *76*, 663−687.

(2) Testa, B.; Carrupt, P. A.; Gaillard, P.; Billois, F.; Weber, P. Lipophilicity in molecular modeling. *Pharm. Res.* **1996**, *13*, 335−343.

(3) Tamai, I.; Tsuji, A. Transporter-mediated permeation of drugs across the blood-brain barrier. *J. Pharm. Sci.* **2000**, *89*, 1371−1388.

(4) Rice, M. E. Ascorbate regulation and its neuroprotective role in the brain. *TINS* **2000**, *23*, 209−216.

(5) Hornig, D. Distribution of ascorbic acid, metabolites and analogues in man and animals. *Ann. New York Acad. Sci.* **1975**, *258*, 103−117.

(6) Lam, D. K. C.; Daniel, P. M. The influx of ascorbic acid into the rat's brain. *Q. J. Exp. Physiol.* **1986**, *71*, 483−489.

(7) Agus, D. B; Gambhir, S. S.; Pardridge, W. M.; Spielholz, C.; Baselga, J.; Vera, J. C.; Golde, D. W. Vitamin C crosses the blood-brain barrier in the oxidized form through the glucose transporters. *J. Clin. Invest.* **1997**, *100*, 2842−2848.

(8) Tsukaguchi, H.; Tokui, T.; Mackenzie, B.; Berger, U. V.; Chen, X. Z.; Wang, Y.; Brubaker, R. F.; Hediger, M. A. A family of mammalian Na^+ -dependent L-ascorbic acid transporters. *Nature* **1999**, *399*, 70−75.

(9) Spector, R.; Lorenzo, A. V. Ascorbic acid homeostasis in the central nervous system. *Am. J. Physiol.* **1973**, *255*, 757−763.

(10) Rajan, D. P.; Huang, W.; Dutta, B.; Devoe, L. D.; Leibach, F. H.; Ganapathy, V.; Prasad, P. D. Human placental sodium-dependent vitamin C transporter (SVCT2): molecular cloning and transport function. *Biochem. Biophys. Res. Comm.* **1999**, *262*, 762−768.

(11) Rumsey, S. C.; Welch, R.W.; Garraffo, H. M.; Ge, P.; Lu, S. F.; Crossman, A. T.; Kirk, K. L.; Levine, M. Specificity of ascorbate analogues for ascorbate transport. Synthesis and detection of [(125)I]6-deoxy-6-iodo-L-ascorbic acid and characterization of its ascorbate-specific transport properties. *J. Biol. Chem.* **1999**, *274*, 23215−23222.

(12) Kim, Y. C.; Zhao, L. X.; Kim, T. H.; Je, S. M.; Kim, E. K.; Choi, H.; Chae, W. G.; Park, M.; Choi, J.; Jahng, Y.; Lee, E. S. Design and synthesis of anticonvulsive agents as gamma-vinylGABA-based potential dual acting prodrugs and their biological activities. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 609−614.

(13) Spath, E. Constitution of kynurenic acid. *Monatsh.* **1921**, *42*, 89−95.

(14) Kato, K.; Terao, S.; Shimamoto, N.; Hirata, M. Studies on scavengers of active oxygen species. 1. Synthesis and biological activity of 2-O-alkylascorbic acids. *J. Med. Chem.* **1988**, *31*, 793−798.

(15) Manfredini, S.; Baraldi, P. G.; Bazzanini, R.; Bortolotti, F.; Vertuani, S.; Ashida, N.; Machida, H. Enzymatic synthesis of 2′-O-acyl-prodrugs of 1-(beta-D-arabinofuranosyl)-5(E)−(2-bromovinyl)uracil (sorivudine, BV-araU) and of 2′-O-acyl-araU, -araC and -araA. *Antiv. Chem. Chemother.* **1998**, *25*, 9−16.

(16) Beuckmann, C. T.; Gordon, W. C.; Kanaoka, Y.; Eguchi, N.; Marcheselli, V. L.; Gerashchenko, D. Y.; Urade, Y.; Hayaishi, O.; Bazan, N. G. Lipocalin-type prostaglandin D synthase (beta-trace) is located in pigment epithelial cells of rat retina and accumulates within interphotoreceptor matrix. *J. Neurosci.* **1996**, *16*, 6119−6124.

(17) Lambert, D. M. Rationale and applications of lipids as prodrug carriers. *Eur. J. Pharm. Sci.* **2000**, *2*, S15−27.

(18) Hodgkins, P. S.; Schwarcz, R. Metabolic control of kynurenic acid formation in the rat brain. *Dev. Neurosci.* **1998**, *20*, 408−416.

(19) Smith, Q. R. Transport of glutamate and other amino acids at the blood-brain barrier. *J. Nutr.* **2000**, *130*, 1016S−1022S.

(20) Hull, M.; Lieb, K.; Fiebich, B. L. Antiinflammatory drugs: a hope for Alzheimer's disease? *Expert Opin. Invest. Drugs* **2000**, *9*, 671−83.

(21) Baures, P. W.; Oza, V. B.; Peterson, S. A.; Kelly, J. W. Synthesis and evaluation of inhibitors of transthyretin amyloid formation based on the nonsteroidal antiinflammatory drug,flufenamic acid. *Bioorg. Med. Chem.* **1999**, *7*, 1339−1347.

(22) Patel, P. M.; Drummond, J. C.; Sano, T.; Cole, D. J.; Kalkman, C. J.; Yaksh, T. L. Effect of ibuprofen on regional eicosanoid production and neuronal injury after forebrain ischemia in rats. *Brain Res.* **1993**, *614*, 315−324.

(23) Frey, H. H.; Popp, C.; Loscher W. Influence of inhibitors of the high affinity GABA uptake on seizure thresholds in mice. *Neuropharmacology* **1979**, *18*, 581−590.

JM015556R