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Synthesis and biological evaluation of phloridzin analogs as human concentrative nucleoside transporter 3 (hCNT3) inhibitors

Amol Gupte, John K. Buolamwini *

Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Sciences Center, 847 Monroe Avenue Suite 327, Memphis, TN 38163, USA

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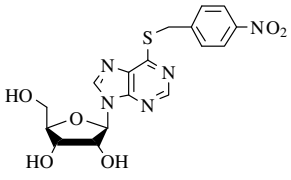
ABSTRACT

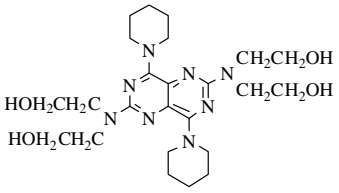
Nucleoside transporter inhibitors have potential therapeutic applications as anticancer, antiviral, cardioprotective and neuroprotective agents. Although quite a few potent inhibitors of the equilibrative nucleoside transporters are known, largely missing are the concentrative nucleoside transporter inhibitors. Phloridzin (**3**, K_i = 16.00 μM) is a known moderate inhibitor of the concentrative nucleoside transporters. We have synthesized and evaluated analogs of phloridzin at the hCNT3 nucleoside transporter. Within the series of synthesized analogs compound **16** (K_i = 2.88 μM), possessing a ribofuranose sugar unit instead of a glucopyranose as present in phloridzin, exhibited the highest binding affinity at the hCNT3 transporter. Phloridzin and compound **16** have also been shown to be selective for the hCNT3 transporter as compared with the hENT1 transporter. Compound **16** can serve as a new lead which after further modifications could yield selective and potent hCNT3 inhibitors.

Hydrophilic nucleosides and their synthetic analogs are transported across cell membranes by integral membrane glycoproteins termed nucleoside transporters.[1] Human nucleoside transporters are classified into two main categories, namely, equilibrative nucleoside transporters (hENTs)[2] and concentrative nucleoside transporters (hCNTs).[3] The potential of nucleoside transporter inhibitors for therapeutic application in cancer chemotherapy,[4] viral infections,[5] inflammatory diseases,[6] cardioprotection,[7] and neuroprotection[8] has been widely discussed. To date, quite a few potent nucleoside transporter inhibitors of the hENTs have been reported. Several analogs of S^6-nitrobenzyl mercaptopurine riboside (NBMPR, **1**), a potent inhibitor of the hENT1, have been synthesized and studied in detail.[9–11] The pyrimidopyrimidine dipyridamole (**2**), and its analogs are also well known inhibitors of both hENT1 and hENT2 nucleoside transporter.[12] However, unlike the hENTs, there is a paucity of potent inhibitors of the hCNTs. Apart from their application in therapeutics, selective and potent hCNT inhibitors will aid in better understanding of the physiological and pathological roles of hCNTs.

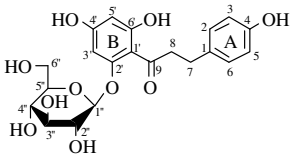
Four isoforms of hENTs have been identified.[13–16] Two isoforms, hENT1[13] and hENT2[14] have been well characterized. Six human CNTs have been identified and they can be classified on the basis of their substrate specificities. These are hCNT1/N2/*cit* (accepts thymidine as a substrate),[17] hCNT2/N1/*cif* (accepts formycin as a substrate),[18] hCNT3/N3/*cib* (exhibits broad substrate specificity),[19] N4/*cit* (like hCNT2 but also accepts guanosine as a permeant),[20] N5/*cs* (sensitive to NBMPR),[20] and N6/*csg* (sensitive to NBMPR and accepts guanosine as a substrate).[20] Of these, only three human isoforms, hCNT1 (SLC28A1),[17] hCNT2 (SLC28A2, also termed SPNT)[18] and hCNT3 (SLC28A3)[19] have been cloned and characterized.



NBMPR, **1**



Dipyridamole, **2**



Phloridzin, **3**

Phloridzin (**3**), a dihydrochalcone glucoside exerts low to moderate inhibitory activity against the three characterized concentrative nucleoside transporters. It inhibits hCNT1 at an IC_{50} of 250 μM, hCNT2 at an IC_{50} of 100 μM, and hCNT3 at a K_i value of 16 μM.[21] The studies conducted in this paper are an effort to address the un-

* Corresponding author. Tel.: +1 901 448 7533; fax: +1 901 448 6828.
E-mail address: jbuolamwini@utmem.edu (J.K. Buolamwini).

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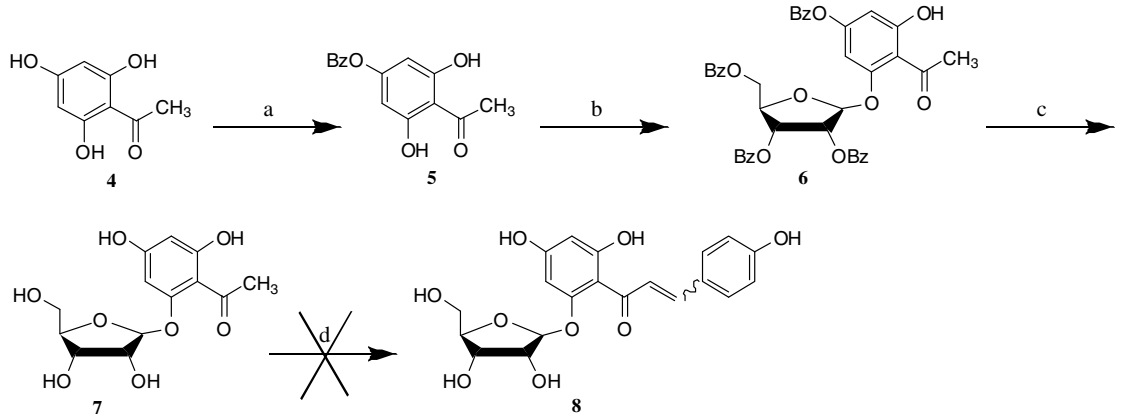
Table 1
Binding affinities (K_i values) of phloridzin and its analogs at the hCNT3 transporter

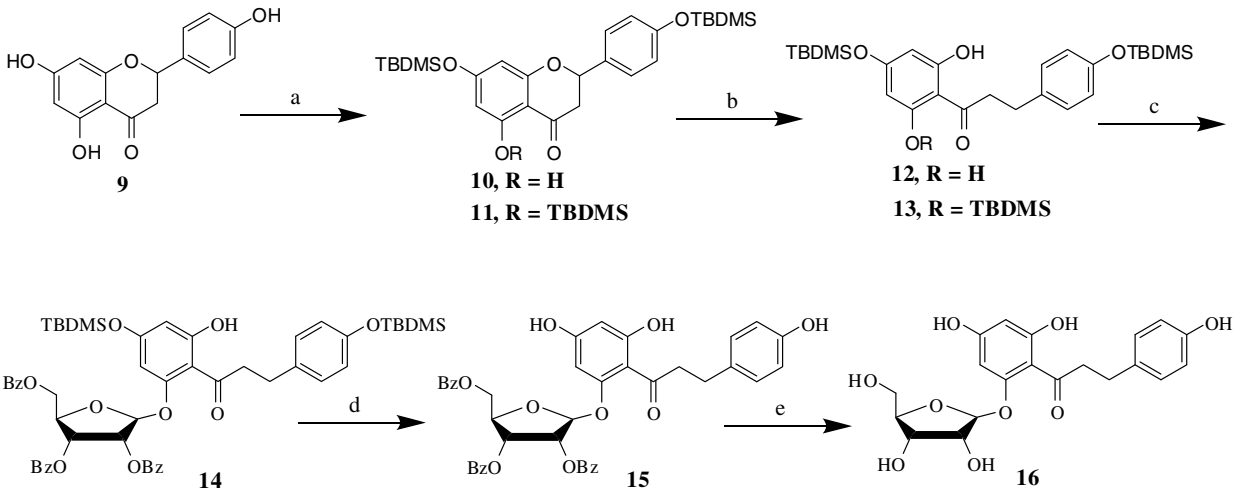
Compound	K_i[a] (μM)
NBMPR (**1**)	88.05 ± 0.03
Phloridzin (**3**)	16.00 ± 0.01
4	>100
7	12.40 ± 0.01
16	2.88 ± 0.01
18	>100
19	>100
20	>100
21	>100
22	>100
23	42.12 ± 0.02
24	>100
25	53.86 ± 0.01
26	30.77 ± 0.01
27	>100
28	>100
29	32.28 ± 0.01
30	>100
31	12.35 ± 0.01
32	30.02 ± 0.01

[a] Binding affinities (K_i values) of phloridzin and its analogs at the hCNT3 transporter determined using [^{3}H]-uridine (K_m = 1.10 ± 0.20 μM)[43] uptake assay in PK15NTD cell line stably transfected with CNT3 cDNA. The results are presented as K_i values ± SEM.

met need of concentrative nucleoside transporter inhibitors, by synthesizing analogs of phloridzin that incorporate specific structural modifications in an attempt to improve the binding affinity of phloridzin at hCNTs. Structure–activity relationship (SAR) studies explored in this paper involve modifications at both the sugar and aglycone subunits of phloridzin. Modifications at the sugar subunit explores the importance of the nature, presence, and position of this subunit. Detailed structure–activity relationship studies on NBMPR a potent hENT1 inhibitor have indicated, among other aspects, a preference of ribofuranose as the sugar subunit over any other sugar moieties.[22] Hence in this study, we have explored the effects of replacing the glucopyranose of phloridzin with a ribofuranose. Modifications at the aglycone subunit of phloridzin focus on the importance of the A- and B-aromatic rings, the presence of hydroxyl groups on these aromatic rings, and the importance of the keto functionality in the linker subunit. These synthesized phloridzin analogs were assayed at the hCNT3 transporter using [^{3}H]-uridine uptake studies in PK15NTD cells expressing the recombinant hCNT3.[21] Phloridzin and the most potent hCNT3 inhibitor within the series of synthesized analogs were also evaluated for their hENT1 inhibitory activity using a flow cytometry assay to gain insights into their selectivity. We present the synthesis and biological evaluation of these new phloridzin analogs as hCNT3 inhibitors (see Table 1).



Scheme 1. Synthesis of compound **7**. Reagents and conditions: (a) BzCl, 2.1 M aq. KOH, −10 °C; (b) ribofuranosyl bromide, K_2CO_3, Acetone; (d) NaOMe, MeOH, reflux; (e) *p*-hydroxybenzaldehyde, KOH or HCl.



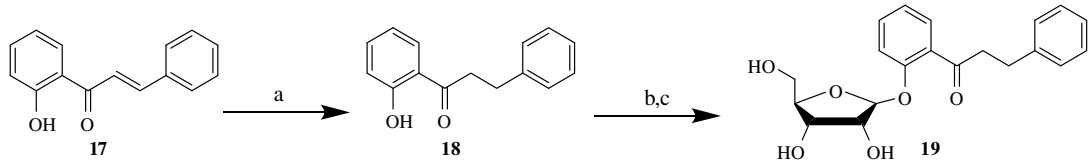
Scheme 2. Synthesis of compound **16**. Reagents: (a) TBDMS-Cl, imidazole, THF; (b) Pd/10%C, HCOOH, HCOONa, 2-propanol; (c) **9**, NaH, THF; (d) TBAF, THF; (e) NaOMe, MeOH, reflux.

In an attempt to replace the glucopyranosyl subunit of phloridzin with a ribofuranose, a solution of 2, 4, 6-trihydroxyacetophenone (**4**) in aqueous KOH was treated with benzoyl chloride (Scheme 1) to obtain the desired 4-hydroxy protected acetophenone (**5**). The other key intermediate, ribofuranosyl bromide, was synthesized from the commercially available β-D-ribofuranose 1-acetate 2, 3, 5-tribenzoate using a published procedure.[23] Successful coupling of this benzoate protected ribofuranosyl bromide with compound **5** was achieved in the presence of potassium carbonate to give compound **6** that on deprotection yielded compound **7**. However, an attempt to synthesize compound **8** using aldol condensation with *para*-hydroxy benzaldehyde under both acidic and basic conditions, proved unsuccessful. Under the acidic aldol conditions a cleavage at the ether linkage between the sugar and aglycone subunits was observed. Hence an alternative synthetic route to replace the glucopyranosyl subunit of phloridzin with a ribofuranose was sought. However, compound **7** synthesized in Scheme 1 was evaluated for its hCNT3 inhibitory activity.

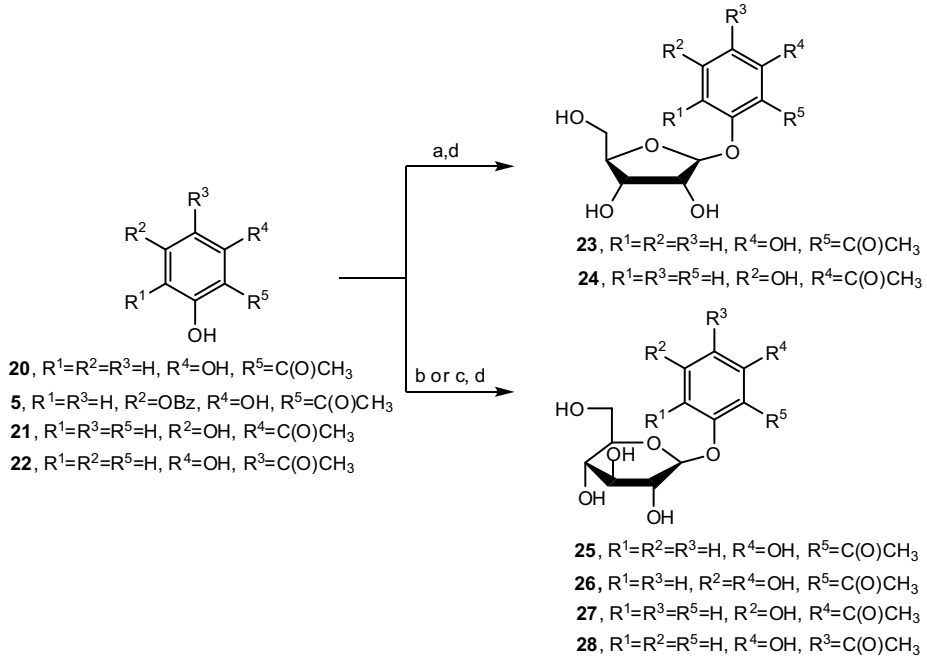
In a successful synthesis (Scheme 2) of the ribofuranosyl analog (**16**), 2′, 4′, 4-trihydroxy flavanone (**9**) was protected using TBDMS to yield the desired dihydroxy protected flavanone (**10**) along with some trihydroxy protected flavanone (**11**). An intramolecular hydrogen bond between the 2′-hydroxyl group and the keto functionality of the flavanone aids in preferential synthesis of the desired compound **10** over compound **11**. Compounds **10** and **11** obtained as an inseparable mixture were subjected to catalytic hydrogenation[24] to yield their corresponding ring opened compounds **12** and **13** that could now be easily separated using column chromatography. Subsequently, compound **12** was successfully coupled with the benzoate protected ribofuranosyl bromide in the presence of NaH to yield compound **14**. Sequential removal of the TBDMS and benzoate protections yielded the desired ribofuranosyl analog (**16**).

Using similar coupling and deprotection strategies, ribofuranosyl analogs **19**, **23,** and **24** were synthesized from their appropriate aglycones as shown in Schemes 3 and 4 to study the importance of the hydroxyl groups present on the aglycone subunit. To compare the activities of these synthesized ribofuranosyl analogs with their corresponding glucopyranosyl analogs, glucopyranosides of 2, 6-dihydroxyacetophenone (**20**) and 3, 5-dihydroxyacetophenone (**21**) were synthesized by phase transfer catalyzed glycosylations with 2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide[25] using benzyltributylammonium chloride as the phase transfer reagent (Scheme 4). Deprotection of the acetyl protection yielded compounds **25** and **27**. The glucopyranoside counterpart of compound **7** was synthesized by coupling compound **5** with 2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide under Koenigs–Knorr conditions for glycosylation[26] followed by deprotection to yield compound **26** (Scheme 4). Phase transfer catalysis was not used for the synthesis of compound **26** as the benzoate protection on compound **5** was lost under these glycosylation conditions.

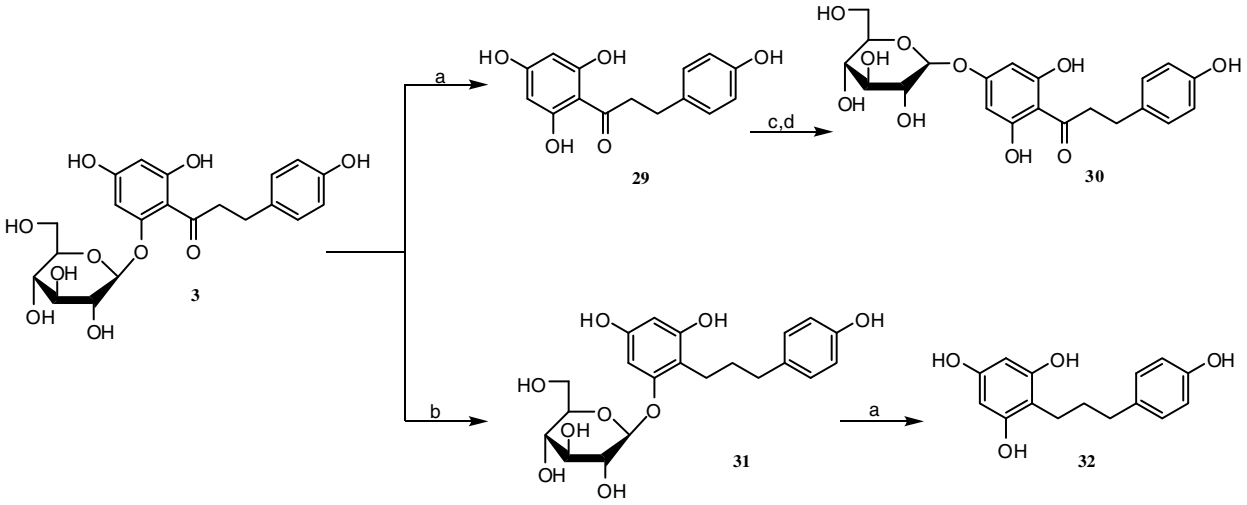
To investigate the importance of the keto functionality on the linker between the aromatic rings A and B, phloridzin was reduced to compound **31** (Scheme 5) using triethylsilane that on deglycosylation yielded compound **32**. Phloridzin was also deglycosylated to yield its corresponding aglycone, phloretin (**29**). Additionally, com-



Scheme 3. Synthesis of compounds **18** and 19. Reagents: (a) H_2 (gas), 2.5 bar, Pd/10%C, MeOH; (b) **9**, NaH, THF; (c) NaOMe, MeOH, reflux.



Scheme 4. Synthesis of compounds **23**–**28**. Reagents: (a) **9**, NaH, THF; (b) 2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, $BnBu_3NCl$, H_2O–$CHCl_3$; (c) 2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, Ag_2O, quinoline; (d) NaOMe, MeOH, reflux.



Scheme 5. Synthesis of compounds **29**–**32**. Reagents and conditions: (a) Ethanol, 1.25 N HCl, 90 °C; (b) Et_3SiH, CF_3COOH; (c) 2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, Ag_2O, quinoline; (d) NaOMe, MeOH, reflux.

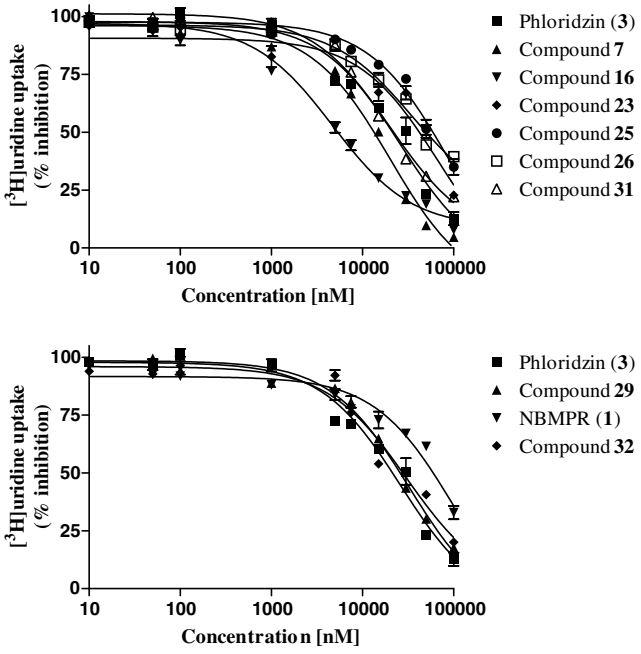


Figure 1. Inhibition of [^{3}H]-uridine uptake by phloridzin and its analogs in PK15NTD cell line stably transfected with CNT3. Cells were incubated with varying concentrations of the inhibitors for 10 min at room temperature. This was followed by a 2-min uptake with [^{3}H]-uridine. The uridine present extracellularly was washed and the cells were lysed using Triton X-100. The radioactivity was measured using a β-scintillation counter.

pounds **28** (Scheme 4) and **30** (Scheme 5) were synthesized using Koenigs–Knorr conditions to investigate the effect of a change in the sugar subunit position on its inhibitory activity at the hCNT3. In both these compounds, glycosylation was selective at the 4′-hydroxyl group of the B-aromatic ring. To understand the importance of the presence of sugar subunits for the hCNT3 inhibitory activity all aglycones were assayed.

The binding affinities of phloridzin (**3**) and its synthesized analogs were studied using a competitive [^{3}H]-uridine uptake assay (Fig. 1)[21] performed on a PK15NTD cell line stably transfected with the hCNT3 cDNA. The synthesized phloridzin analogs displayed binding affinities ranging from low micromolar concentrations for compound **16** (K_i = 2.88 μM) to high micromolar concentrations for compounds **19**, **24**, **27**, **28**, and **30** (K_i > 100 μM).

A 5.5-fold improvement in activity has been observed for compound **16** (K_i = 2.88 μM) compared to phloridzin (**3**, K_i = 16.00 μM). A marginal 1.3-fold improvement in activity over phloridzin is seen for compound **7** (K_i = 12.40 μM) although it lacks the A ring in the aglycone subunit. The lower K_i values of compounds **7** and **16** when compared to phloridzin indicate that replacing the glucopyranose with a ribofuranose results in an improvement in binding of these analogs at the hCNT3. A similar trend of the ribofuranose being better than the glucopyranose is evident on comparing the binding affinities of compound **7** (K_i = 12.40 μM) with compound **26** (K_i = 30.77 μM), a difference of about 2.5-fold, and compound **23** (K_i = 42.12 μM) with compound **25** (K_i = 53.86 μM), a difference of about 1.3-fold. Although the improvement in binding affinity of most ribofuranose analogs over corresponding glucopyranose analogs is modest, a consistent trend of the ribofuranose substitution being better than glucopyranose is observed. The binding affinity of the aglycone phloretin (**29**, K_i = 32.28 μM) at the hCNT3 is about 2-fold lower than that of phloridzin (**3**) and about 11-fold lower than that of compound **16** indicating that the presence of either a glucopyranose or a ribofuranose at the 2′-hydroxyl improves binding affinity. Additionally, the ribofuranosyl analog, compound **7** (K_i = 12.40 μM) and its corresponding glucopyranosyl analog, compound **26** (K_i = 30.77 μM) display an improved binding affinity over their aglycone subunit compound **4** (K_i > 100 μM). A similar trend is also observed in the case of compound **23** (K_i = 42.12 μM) and compound **25** (K_i = 53.86 μM) when compared to their aglycone subunit (**20**, K_i > 100 μM). Compound **31** (K_i = 12.35 μM), that possesses a glucopyranosyl subunit at the 2′-hydroxyl of deoxyphloretin (**32**, K_i = 30.06 μM), also displays a marginally improved binding affinity at the hCNT3. These results show that the presence of a sugar subunit at the 2′-hydroxyl of the aglycone subunits leads to an improvement in the binding affinity at the hCNT3. Moving the sugar subunit, from the 2′-hydroxyl to the 4′-hydroxyl, leads to a major loss of binding affinity at hCNT3. This is confirmed by the binding affinities of compounds **28** and **30**, which exhibit K_i values greater than 100 μM. Compounds **24** and **27** also highlight the preference of the 2′-hydroxyl position for the sugar as both these compounds exhibit K_i values greater than 100 μM.

Interestingly, it appears that the keto group on the linker of the aglycone subunit does not play a significant role in the binding of these compounds to hCNT3, as there was a very small difference between compounds possessing this functional group and those that lack it. A comparison of compound **31** (K_i = 12.35 μM) with phloridzin (**3**, K_i = 16.00 μM) and compound **32** (K_i = 30.02 μM)

with phloretin (**37,** K_i = 32.28 μM) highlights this observation. Additionally, the presence of hydroxyl groups on the aromatic aglycone subunit of the phloridzin scaffold is important for high binding affinities at the hCNT3. Removal of the hydroxyl groups present on the A and B rings leads to a substantial loss in binding affinity. This is exemplified by the binding affinity of compound **19** (K_i > 100 μM), which has a structure similar to phloridzin but lacks the 4′-, 6′- and 4-position hydroxyl groups on the aromatic A and B rings. Also, compounds possessing both the A and B rings in their aglycone subunits bind better than their corresponding analogs possessing just the B ring. A comparison between phloridzin (**3**, K_i = 16.00 μM) with compound **26** (K_i = 30.77 μM) and compound **16** (K_i = 2.88 μM) with compound **7** (K_i = 12.40 μM) reveals this trend.

In addition to the [^{3}H]-uridine uptake experiments, a flow cytometric competitive binding assay was also undertaken to gain knowledge regarding the selectivity of these analogs.[27] Although NBMPR (**1**) inhibits hCNT3 with a K_i of 88.05 μM it is known to bind the hENT1 nucleoside transporter in subnanomolar concentrations (K_i = 0.7 nM). The most active hCNT3 inhibitor in this series, compound **16** (K_i = 2.88 μM), and the prototype, phloridzin (**3**) (K_i = 16.00 μM), exhibited K_i values of >1 mM at the hENT1 thus indicating that these compounds are highly selective for hCNT3 relative to hENT1.

Contrary to hENTs, the knowledge regarding inhibitors of hCNTs has been strikingly limited. In this SAR study, we have introduced specific modifications in the phloridzin scaffold while demonstrating the importance of a sugar subunit at the 2′-hydroxyl position for exhibiting binding affinity at the hCNT3. Replacing the glucopyranose of phloridzin with a ribofuranose, results in a binding improvement at the hCNT3. Presence of hydroxyl groups on the aromatic A and B rings is important for binding at the hCNT3. Additionally, phloridzin and its most potent analog, compound **16** do not exhibit significant binding affinity at the hENT1 (K_i > 1 mM), highlighting their selectivity for hCNT3 when compared with hENT1. The knowledge gained from this study will be significant in our efforts in developing selective high affinity ligands of the hCNT3 transporter. This study identifies compound **16** as a potential lead that may be modified to yield more selective and potent hCNT3 inhibitors.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.112.

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