Differential Inhibition of Equilibrative Nucleoside Transporter 1 (ENT1) Activity by Tyrosine Kinase Inhibitors

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Abstract
Background and Objectives Equilibrative nucleoside transporter (ENT) 1 is a widely-expressed drug transporter, handling nucleoside analogues as well as endogenous nucleosides. ENT1 has been postulated to be inhibited by some marketed tyrosine kinase inhibitors (TKIs). To obtain insights into this point, the interactions of 24 TKIs with ENT1 activity have been analyzed.

Methods Inhibition of ENT1 activity was investigated in vitro through quantifying the decrease of [3H]-uridine uptake caused by TKIs in HAP1 ENT2-knockout cells, exhibiting selective ENT1 expression. TKI effects towards ENT1-mediated transport were additionally characterized in terms of the in vivo relevance and of their relationship to TKI molecular descriptors. Putative transport of the TKI lorlatinib by ENT1/ENT2 was analyzed by LC-MS/MS.

Results Of 24 TKIs, 12 of them, each used at 10 µM, were found to behave as moderate or strong inhibitors of ENT1, i.e., they decreased ENT1 activity by at least 35%. This inhibition was concentration-dependent for at least the strongest ones (IC50 less than 10 µM) and was correlated with some molecular descriptors, especially with atom-type E-state indices. Lorlatinib was notably a potent in vitro inhibitor of ENT1/ENT2 (IC50 values around 1.0–2.5 µM) and was predicted to inhibit these nucleoside transporters at relevant clinical concentrations, without, however, being a substrate for them.

Conclusion Our data unambiguously add ENT1 to the list of drug transporters inhibited by TKIs, especially by lorlatinib. This point likely merits attention in terms of possible drug–drug interactions, notably for nucleoside analogues, whose ENT1-mediated uptake into their target cells may be hampered by co-administrated TKIs such as lorlatinib.

1 Introduction

Equilibrative nucleoside transporter (ENT) 1 (SLC29A1) is a major plasma membrane transporter of nucleosides, widely expressed in various cell types [1]. It mediates facilitated diffusion of both purine (adenosine, guanosine, and inosine) and pyrimidine (cytidine, thymidine, and uridine) nucleosides into cells, in a sodium-independent manner [2]. Such substrates are shared by ENT2 (SLC29A2), another member of the ENT subfamily, also ubiquitously expressed, but with an average expression level lower than that of ENT1, and exhibiting low sensitivity to the potent ENT1 inhibitor, nitrobenzylmercaptopurine riboside (NBMPR) [3].

Besides physiological nucleosides, ENT1 transports nucleosides-derived drugs, used for the treatment of cancers, inflammatory diseases, or viral infections [4]. In this way, ENT1 is directly implicated in the uptake of anticancer or antiviral drugs into target cells, and, consequently, in their pharmacological effects. ENT1 activity is thus a key determinant of the anticancer activity of gemcitabine in pancreatic tumor cells [5]. Decreased expression and/or activity of ENT1 can therefore result in cellular resistance to cytotoxic nucleoside analogues through reduction of their intracellular accumulation [6, 7]. ENT1 may also be implicated in pharmacokinetics, notably at the hepatic level, and at the placental and blood–testis barriers [8–10]. ENT1 additionally deserves recent attention in the context of coronavirus disease 2019 (COVID-19). Indeed, it may transport nucleoside analogue candidates for inhibiting Severe Acute Respiratory Syndrome CoronaVirus-2 (SARS-CoV-2) replication...
Inhibition of equilibrative nucleoside transporter (ENT1) activity by 24 marketed or under clinical development tyrosine kinase inhibitors (TKIs) was investigated in vitro using cells selectively expressing ENT1.

Of 24 TKIs, 12 of them, used at 10 µM, behaved as moderate or strong inhibitors of ENT1-mediated uridine transport, with notably one (lorlatinib) exerting potent in vitro inhibition (IC50 = 2.5 µM) and predicted to inhibit ENT1 activity at clinically achievable plasma concentrations, without, however, being an ENT1 substrate.

These data indicate that TKIs, especially lorlatinib, may have to be considered for possible ENT1-based drug–drug interactions (DDIs), notably with known ENT1 substrates like nucleoside analogues.

2 Materials and Methods

2.1 Chemicals

The 22 marketed TKIs, abemaciclib, acalabrutinib, afatinib, alectinib, brigatinib, cabozantinib, ceritinib, crizotinib, dacomitinib, entrectinib, ibrutinib, lapatinib, lenvatinib, lorlatinib, neratinib, nintedanib, osimertinib, regorafenib, ribociclib, ruxolitinib, tofacitinib, and vemurafenib, as well as itacitinib and pacritinib, 2 TKIs under clinical phase 3 development, were provided by MedChemtronica (Sollen-tuna, Sweden). These TKIs target various kinases, as indicated in Table 1. Stock solutions of TKIs were initially prepared using dimethylsulfoxide as solvent. NBMPR was from Sigma-Aldrich (Saint-Quentin Fallavier, France) whereas [5,6-3H]-uridine (specific activity = 30 Ci/mmol) was from Perkin-Elmer (Villebon-sur-Yvette, France).

2.2 Cell Culture

HAP1 wild-type cells (reference C631) and CRISPR-Cas9-edited HAP1 knockout (KO) cells deficient for ENT1 (HAP1 ENT1-KO cells; reference HZGHC000785c004) or for ENT2 (HAP1 ENT2-KO cells; reference HZGHC005532c010) were from Horizon Discovery (Watertown, UK). HAP1 wild-type cell line is a human near-haploid cell line derived from the male chronic myelogenous leukemia cell line KBM-7 [20]. HAP1 wild-type and KO cells were routinely cultured in Iscove’s modified Dulbecco’s medium (Thermo Fisher Scientific) supplemented with 10% (vol/vol) fetal calf serum, 20 IU/mL penicillin, and 20 μg/mL streptomycin. Lung A549 cells were routinely cultured in F-12K medium, supplemented with 2 mM L-glutamine, 10% fetal calf serum (vol/vol), 20 IU/mL penicillin, and 20 μg/mL streptomycin, as previously described [21]; the A549 cell line is a human cancerous alveolar basal epithelial cell line, established from a pulmonary adenocarcinoma of a 58-year-old Caucasian male [22].

2.3 Nucleoside Transport Assays

Cells previously seeded in 96-well plates were incubated with 8.33 nM [3H]-uridine for 5 min at 37 °C, in a defined sodium-free transport assay buffer, consisting of 5.3 mM KCl, 1.1 mM KH2PO4, 0.8 mm MgSO4, 1.8 mM CaCl2, 11
**Table 1** Effect of 10 µM TKIs on ENT1 activity in HAP1-ENT2 KO cells.

| TKI                  | Main targeted kinase | % inhibition of ENT1 activity (mean ± SD) |
|----------------------|----------------------|------------------------------------------|
| Abemaciclib          | CDK4/6               | 25.0 ± 7.9**                            |
| Acalabrutinib        | BTK                  | 42.6 ± 10.4***                           |
| Afatinib             | EGFR                 | 54.5 ± 2.4***                            |
| Allecitinib          | ALK                  | 54.0 ± 4.9***                            |
| Brigatinib           | ALK                  | 52.5 ± 11.1***                           |
| Cabozantinib         | VEGFR2, c-Met, c-Kit, Axl and Flt3 | 48.8 ± 2.5***                           |
| Ceritinib            | ALK                  | 26.3 ± 1.7**                             |
| Crizotinib           | ALK                  | 34.7 ± 2.7**                             |
| Dacomitinib          | EGFR                 | 43.9 ± 2.3***                            |
| Entrectinib          | Pan-TRK, ROS1, and ALK | 22.9 ± 5.2*                             |
| Ibrutinib            | BTK                  | 72.7 ± 1.1***                            |
| Itacitinib           | JAK1                 | 21.9 ± 13.3*                             |
| Lapatinib            | ErbB-2 and EGFR      | 25.7 ± 15.2**                            |
| Lenvatinib           | VEGFR1-3, FGFR1-4, PDGFR, Kit, and Ret | 63.1 ± 5.9***                           |
| Lorlatinib           | ROS1 and ALK         | 86.6 ± 6.4***                            |
| Neratinib            | ErbB-2 and EGFR      | 61.0 ± 8.5***                            |
| Nintedanib           | VEGFR, FGFR and PDGFR | 46.9 ± 9.6***                            |
| Osimertinib          | EGFR                 | 32.3 ± 11.3***                           |
| Pacritinib           | JAK2 and Flt3        | 61.6 ± 8.1***                            |
| Regorafenib          | VEGFR1/2/3, PDGFRβ, Kit, Ret and Raf-1 | 21.4 ± 2.7*                             |
| Ribociclib           | CDK4/6               | 17.8 ± 10.2                              |
| Ruxolitinib          | JAKs                 | 34.1 ± 13.2***                           |
| Tofacitinib          | JAKs                 | 4.8 ± 8.3                                |
| Vemurafenib          | B-Raf                | 27.3 ± 10.9***                           |

Experimental data are the means ± SD of three independent assays. ENT1 activity in control HAP1-ENT2 KO cells not exposed to TKIs cells is 2.1 ± 0.2 pmol uridine/mg protein/5 min

**TKI** anaplastic lymphoma kinase, **BTK** Bruton’s tyrosine kinase, **c-Met** hepatocyte growth factor receptor (HGFR), **CDK** cyclin-dependent kinase, **EGFR** epidermal growth factor receptor (also known as ErbB-1 or HER1), **ErbB-2** human epidermal growth factor receptor-2 (also known as HER2), **FGFR** fibroblast growth factor receptor, **Flt3** Fms-like tyrosine kinase 3, **JAK** Janus kinase, **PDGFR** platelet-derived growth factor, **TRK** tropomyosin receptor kinase (also known as NTRK, neurotrophic tyrosine receptor kinase), **VEGFR** vascular endothelial growth factor, **TKIs** tyrosine kinase inhibitors

*p < 0.05, **p < 0.01, and ***p < 0.001, when compared to control cells not exposed to TKIs

mM α-glucose, 10 mM Hepes, and 136 mM N-methyl-glucamine, and adjusted to pH 7.4, in the absence or presence of NBMPR (used at 0.1 or 100 µM for inhibiting ENT1 or ENT1/ENT2, respectively) or TKI, as previously described [23]. After two washes in ice-cold phosphate-buffered saline (PBS), cells were lysed using distilled water, and cellular uptake of radiolabeled uridine (U) was determined through scintillation counting and normalized to total protein content, determined by the Bradford method [24]. Nucleoside transporter activities in the absence of TKIs were next determined using the following equations (Eqs. 1 and 2):

\[
\text{ENT1 activity} = U_{\text{Control}} - U_{0.1 \mu M \text{NBMPR}} \quad (1)
\]

\[
\text{ENT2 activity} = U_{0.1 \mu M \text{NBMPR}} - U_{100 \mu M \text{NBMPR}} \quad (2)
\]

with \( U_{\text{Control}} \) is the uptake of \([^{3}H]\)-uridine in the absence of NBMPR, \( U_{0.1 \mu M \text{NBMPR}} \) is the uptake of \([^{3}H]\)-uridine in the presence of 0.1 µM NBMPR and \( U_{100 \mu M \text{NBMPR}} \) = uptake of \([^{3}H]\)-uridine in the presence of 100 µM NBMPR.

ENT1 activity in HAP1 ENT2-KO cells in the presence of TKI was expressed as % of ENT1 activity found in control cells or as % of ENT1 activity inhibition according to the following equations (Eqs. 3 and 4):

\[
\text{ENT1 activity} = \left( \frac{U_{\text{TKI}} - U_{0.1 \mu M \text{NBMPR}}}{U_{\text{Control}} - U_{0.1 \mu M \text{NBMPR}}} \right) \times 100 \% \quad (3)
\]

\[
\text{ENT1 activity inhibition} = 100 \% - \text{ENT1 activity}\% \quad (4)
\]
with \( U_{\text{TKI}} \) is the \([3\text{H}]-\text{uridine} \) uptake in the presence of TKI.

ENT2 activity in the presence of TKI in HAP1 ENT1-KO cells was expressed as % of ENT2 activity found in control cells, as described in the Eq. (5):

\[
\text{ENT2 activity} \% = \left( \frac{U_{\text{TKI}} - U_{100 \mu M \text{ NBMPR}}}{U_{\text{Control}} - U_{100 \mu M \text{ NBMPR}}} \right) \times 100.
\]

ENT activity in the presence of lorlatinib in A549 cells was expressed as % of total ENT activity found in control cells using the following equation:

\[
\text{ENT activity} \% = \left( \frac{U_{\text{TKI}} - U_{100 \mu M \text{ NBMPR}}}{U_{\text{Control}} - U_{100 \mu M \text{ NBMPR}}} \right) \times 100.
\]

(6)

Half-maximal inhibitory concentration (IC\(_{50}\)) for TKIs towards ENT1, ENT2, or total ENT activity was calculated using Prism 8.4.2 software (GraphPad Software, La Jolla, CA, USA), through nonlinear regression based on the following four-parameter logistic equation (Eq. 7):

\[
A = \frac{100}{1 + 10^{((I - \log(I_{50})))/\text{Hill slope}}}
\]

(7)

with \( A \) is the percentage of transporter activity for a given concentration of TKI, determined as described in Eqs. (3), (5), or (6), \( I \) is the TKI concentration in the medium, and Hill slope = a coefficient describing the steepness of the curve.

### 2.4 Prediction of In vivo Inhibition of ENT Activity by TKIs

From in vitro IC\(_{50}\) values, in vivo inhibition of ENT activity by TKIs was predicted through applying criteria defined by the US Food and Drug Administration (FDA) guidance on in vitro drug interaction studies [25] to ENT1 or ENT2, i.e., a transporter-related DDI can be expected at a systemic level if (Eq. 8):

\[
I_{\text{max,u}}/I_{\text{IC}_{50}} \geq 0.1
\]

(8)

with \( I_{\text{max,u}} \) is the maximum unbound plasma concentration of the TKI.

### 2.5 Lorlatinib Accumulation Assay

HAP1 wild-type, ENT1-KO, and ENT2-KO cells were incubated with 10 µM lorlatinib, in the absence or presence of 0.1 or 100 µM NBMPR, for 5 min at 37 °C. Cells were then washed twice in ice-cold PBS, lysed in distilled water, and acetonitrile-based extraction of cell lysates was performed. Lorlatinib quantification was next performed through liquid chromatography-tandem mass spectrometry (LC–MS/MS), based on a high-performance liquid chromatography Aria system (Agilent, Les Ulis, France), equipped with a Poroshell 120 C18 (4.6 × 100 mm) column (Agilent) and coupled to a tandem mass spectrometry TSQ Quantum (Thermo Fisher Scientific, Villebon sur Yvette, France), fitted with an electrospray ionization source (ESI+). Monitored ion transitions were at 407 > 228.1 m/z. Data were finally normalized to protein content.

### 2.6 TKI Molecular Descriptor Generation

The total of 829 molecular descriptors belonging to the blocks “constitutional indices” \((n = 47)\), “ring descriptors” \((n = 32)\), “topological indices” \((n = 75)\), “walk and path counts” \((n = 46)\), “connectivity indices” \((n = 37)\), “information indices” \((n = 50)\), “geometrical descriptors” \((n = 38)\), “functional group counts” \((n = 154)\), “atom-centred-fragments” \((n = 115)\), “atom-type E-state indices” \((n = 172)\), “charge descriptors” \((n = 15)\), “molecular properties” \((n = 20)\), and “drug-like indices” \((n = 28)\), were determined for all TKIs using the Dragon 7.0 software (Kode Chemoinformatics, Pisa, Italy) (See https://chm.kode-solutions.net/products_dragon_descriptors.php for a complete list of these descriptors). These compounds, initially expressed in SMILES format, were converted to 3D format using the MarvinView software (ChemAxon, Budapest, Hungary) before processing by Dragon 7.0 software to obtain molecular descriptors, as previously described [26].

### 2.7 Statistical Analysis

Experimental data were routinely expressed as means ± SD of at least three independent assays, each usually being performed in triplicate. Data were statistically analyzed through analysis of variance followed by the Dunnett’s post hoc test or through Spearman’s rank correlation test. The criterion of significance was \( p < 0.05 \).

### 3 Results

ENT activity was first investigated via \([3\text{H}]-\text{uridine} \) uptake in HAP1 wild-type, HAP1-ENT1-KO, and HAP1-ENT2-KO cells. As shown in Fig. 1, HAP1 wild-type cells exhibited ENT1 activity higher than that of ENT2. HAP1-ENT2-KO cells exhibited similar levels of ENT1-mediated \([3\text{H}]-\text{uridine} \) uptake compared to HAP1 wild-type cells, but failed to exhibit notable ENT2 activity (Fig. 1). By contrast, HAP1 ENT1-KO cells displayed similar ENT2 activity compared to HAP1 wild-type counterparts, but ENT1 activity was fully suppressed in these KO cells (Fig. 1). Taken together, these
data indicate that HAP1 ENT-KO cells were fully functional, HAP1 ENT2-KO cells exhibited only ENT1 activity and can thus be used for specifically studying ENT1, whereas HAPI ENT1-KO cells exhibited only ENT2 activity and can therefore be retained for specifically investigating ENT2 activity.

The effects of 10 µM TKIs towards ENT1-mediated transport of [3H]-uridine were next studied in HAP1 ENT2-KO cells. As shown in Table 1, 2/24 TKIs, i.e., ribociclib and tofacitinib, failed to significantly alter ENT1 activity. Among the 22/24 TKIs which inhibited ENT1, some of them (10/24 TKIs), i.e., abemaciclib, ceritinib, crizotinib, entrectinib, itacitinib, lapatinib, osimertinib, regorafenib, ruxolitinib, and vemurafenib, decreased ENT1 activity by less than 35% and can thus be considered as weak ENT1 inhibitors. Other TKIs (7/24 TKIs), i.e., acalabrutinib, afatinib, alec tinib, brigatinib, caboza tinib, dacomitinib, and nindetanib, were found to inhibit ENT1 activity by more than 35% but less than 60% (Table 1), and thus appear as moderate inhibitors of the transporter. Finally, 5/24 TKIs, i.e., ibrutinib, lenvatinib, lorlatinib, neratinib, and pacritinib were found to inhibit ENT1 by more than 60% (Table 1), and are consequently potent inhibitors of ENT1 activity. These five strong ENT1 inhibitors were next found to inhibit ENT1-mediated transport of [3H]-uridine in HEK ENT2-KO cells in a concentration-dependent manner, with IC50 values ranging from 2.5 µM (for lorlatinib) to 8.5 µM (for neratinib) (Fig. 2).

In order to investigate the physico-chemical structural requirements for ENT1 inhibition by TKIs, the percent ages of inhibition of ENT1 activity by the 24 TKIs were next compared to values of 829 TKI molecular descriptors, through Spearman’s rank correlation analysis, and 20/829 molecular descriptors were found to be significantly either positively (n = 13) or negatively (n = 7) correlated with ENT1 inhibition (Table 2). Among these correlated descriptors, the most significant belonged to the blocks “constitutional indices” (Mi, p = 0.005), “functional group counts” (nAROR, p = 0.006) and “Atom-Type E-state indices” (SssO, p = 0.0003, and NssO, p = 0.0006) (Table 2). The molecular descriptors not correlated with ENT1 activity included lipophilicity-related parameters such as the Moriguchi octanol–water partition coefficient and the Ghose–Crippen octanol–water partition, as well as the topological polar surface area (data not shown).

The prediction of in vivo ENT1 inhibition was next performed for TKIs defined above as strong inhibitors, i.e., ibrutinib, lenvatinib, lorlatinib, neratinib, and pacritinib. It was done using the criteria defined by the FDA for systemic inhibition of transporters and based on the ratio Imax,u/IC50 [25]. As indicated in Table 3, only lorlatinib was predicted to inhibit in vivo ENT1 activity at clinically achievable concentrations. We therefore further characterized this interaction of lorlatinib with ENT1. We notably determined whether lorlatinib-mediated inhibition of ENT1 activity described above in engineered HAP1 ENT2-KO cells may also occur in human tumoral cells. For this, we used lung cancer A549 cells, which exhibited a high level of [3H]-uridine uptake inhibited by a low concentration (0.1 µM) of NBMPR, representing 88.9 ± 0.5% of total [3H]-uridine uptake (Fig. 3A). This demonstrates that ENT1 is the preponderant functional ENT isoform in lung A549 cells. Lorlatinib used at 10 µM markedly decreased [3H]-uridine accumulation in A549 cells by 82.5 ± 1.1%. This inhibitory effect of the TKI in A549 cells was demonstrated to be concentration-dependent, with an IC50 of 2.0 µM (Fig. 3B), close to the value reported above for HAP1 ENT2-KO cells. The effects of lorlatinib towards ENT2 activity was next studied in HAP1 ENT1-KO cells. As shown in Fig. 4, lorlatinib potently inhibited ENT2 activity in a concentration-dependent manner, with an IC50 (1.0 µM) even lower than the one measured on ENT1 activity in HAP1 ENT2-KO cells (2.5 µM). With a lorlatinib Imax,u of 485 nM (Table 3), the ratio Imax,u/IC50 for ENT2 inhibition by lorlatinib was consequently 0.48, greater than the threshold of 0.1 given by the FDA guidance on DDIs [24]. This means that this interaction with ENT2 should also be clinically relevant, as predicted above for that with ENT1. Whether lorlatinib may be a substrate for ENT1/ENT2 was finally investigated.

### Fig. 1

**ENT activities in HAP1 wild-type, ENT1-KO, and ENT2-KO cells.** Cells were incubated with [3H]-uridine for 5 min at 37 °C, in the absence or presence of 0.1 µM or 100 µM NBMPR. Intracellular uptake of [3H]-uridine was next determined by scintillation counting and normalized to protein content. Data are expressed as ENT1 or ENT2 activity, according to Eqs. (1) and (2) reported in “Materials and Methods” and are the means ± SD of at least three independent assays. ***p < 0.001, NS not statistically significant, when compared to HAP1 wild-type cells. **ENT equilibrative nucleoside transporter, KO knockout, NBMPR nitrobenzylmercaptopurine riboside**
through comparing the cellular accumulation of the TKI in HAP1 wild-type, ENT1-KO, and ENT2-KO cells, in the absence or presence of 0.1 or 100 µM NBMPR. Lorlatinib was found to similarly accumulate in HAP1 wild-type, ENT1-KO, and ENT2-KO cells, and the presence of 0.1 µM or 100 µM NBMPR failed to reduce cellular uptake of lorlatinib (Fig. 5).

4 Discussion

The present study fully confirms that TKIs can interact with ENT1 activity. Indeed, only 2/24 TKIs were devoid of any inhibitory effect towards ENT1 when used at 10 µM, whereas 12/24 TKIs were characterized as moderate (35–60% inhibition of ENT1 activity) or strong (more than 60% inhibition of ENT1 activity) inhibitors of ENT1 and 10/24 TKIs as weak inhibitors (less than 35% of ENT1 inhibition). In particular, ibrutinib, lenvatinib, lorlatinib, neratinib, and pacritinib were found to potently inhibit ENT1 with relative low IC₅₀ values (ranging from 2.5 to 8.5 µM) and can be unambiguously added to the list of TKIs blocking ENT1 activity, which already comprises 11 other TKIs, including the Bcr-Abl inhibitors, imatinib and nilotinib, and the epidermal growth factor receptor inhibitors, gefitinib and erlotinib [17, 18]. In addition to ENT1, TKIs have already been shown to inhibit various SLCs, handling organic cations, like organic cation transporter (OCT) 1
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**(SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3), multidrug and toxin extrusion protein (MATE) 1 (SLC47A1) and MATE2-K (SLC47A2)** [27, 28], or organic anion transporters, like organic anion-transporting polypeptide (OATP) 1B1 (SLCO1B1) [29]. Sodium-dependent concentrative nucleoside transporters (CNTs) are also inhibited by some TKIs, including CNT2 (SLC28A2) blocked by imatinib [17] and CNT3 (SLC28A3) by erlotinib [18]. In this context, it is noteworthy that the nature of the inhibited SLCs depends on the TKIs. For example, the Janus kinase inhibitor (JAK) 1/2 ruxolitinib rather potently inhibits OCT2, MATE1, MATE2-K, and the organic anion transporter (OAT) 3 (SLC22A8)

### Table 2 TKI molecular descriptors correlated with ENT1 activity inhibition percentage by 10 μM TKIs.

| Molecular descriptor | Block | Name | Correlation with ENT1 inhibition % | Spearman coefficient (ρ) | Significance (p value) |
|----------------------|-------|------|-----------------------------------|--------------------------|----------------------|
| **Constitutional indices** | Mean first ionization potential (scaled on Carbon atom) (Mi) | – 0.56 | 0.005 |
| **Ring descriptors** | Number of 10-membered rings (nR10) | 0.47 | 0.020 |
| | Distance/detour ring index of order 10 (D/Dtr10) | 0.43 | 0.034 |
| **Geometrical descriptors** | Length-to-breadth ratio by WHIM (L/Bw) | – 0.42 | 0.039 |
| **Functional group counts** | Number of total tertiary C(sp3) (nCt) | – 0.42 | 0.041 |
| | Number of ring tertiary C(sp3) (nCrt) | – 0.42 | 0.041 |
| | Number of aliphatic secondary C(sp2) (nR=Cs) | 0.45 | 0.027 |
| | Number of nitriles (aromatic) (nArCN) | 0.45 | 0.029 |
| | Number of ethers (aromatic) (nArOR) | 0.54 | 0.006 |
| **Atom-centred fragments** | =CHR (C-016) | 0.45 | 0.027 |
| | H attached to alpha-C (H-051) | – 0.45 | 0.028 |
| | Al–O–Ar / Ar–O–Ar / R..O..R/R–O–C=X (O-060) | 0.52 | 0.01 |
| **Atom-type E-state indices** | Sum of dsCH E-states (SdsCH) | 0.45 | 0.026 |
| | Sum of sNH2 E-states (SsNH2) | 0.44 | 0.031 |
| | Sum of ssO E-states (SssO) | 0.67 | 0.0003 |
| | Number of atoms of type dsCH (NdsCH) | 0.45 | 0.027 |
| | Number of atoms of type sNH2 (NsNH2) | 0.47 | 0.021 |
| | Number of atoms of type ssO (NsssO) | 0.65 | 0.0006 |
| **Charge descriptors** | Maximum positive charge (qpmax) | – 0.52 | 0.010 |
| | Submolecular polarity parameter (SPP) | – 0.43 | 0.037 |

*ENT* equilibrative nucleoside transporter, *TKIs* tyrosine kinase inhibitors

### Table 3 Prediction of in vivo ENT1 inhibition by TKIs.

| TKI | C<sub>max</sub> | f<sub>u</sub> | I<sub>max,u</sub> | IC<sub>50</sub> | I<sub>max,u</sub>/IC<sub>50</sub> | Predicted in vivo inhibition<sup>a</sup> | Reference |
|-----|----------------|----------|----------------|-------------|----------------|-------------------------------|-----------|
| Ibrutinib | 354 nM | 0.027 | 9.56 nM | 4.5 µM | 0.002 | No | [43] |
| Lenvatinib | 761 nM | 0.016 | 12.02 nM | 7.4 µM | 0.002 | No | [44] |
| Lorlatinib | 1466 nM | 0.34 | 485 nM | 2.5 µM | 0.194 | Yes | [45] |
| Neratinib | 146 nM | 0.01 | 1.46 nM | 8.5 µM | <0.001 | No | [46] |
| Pacritinib | 22500 nM | 0.011 | 247.5 nM | 3.7 µM | 0.067 | No | [47] |

<sup>c<sub>max</sub></sup> maximum plasma concentration, f<sub>u</sub> unbound fraction, I<sub>max,u</sub> maximum unbound plasma concentration, *ENT* equilibrative nucleoside transporter, *TKIs* tyrosine kinase inhibitors

<sup>a</sup> An in vivo inhibition can be predicted if the ratio I<sub>max,u</sub>/IC<sub>50</sub> ≥ 0.1 according to the US FDA guidance criteria [25] applied to ENT1
(with IC_{50} values less than 10 µM), whereas it only weakly decreases ENT1 activity and fails to alter that of OCT1 [30]. In the same way, ceritinib and crizotinib are potent inhibitors of OCT3 (with IC_{50} values in the range 30–40 nM) [28], but only modestly decrease ENT1 activity when used at 10 µM (Table 1). Such data indicate that any general, unspecific and uniform inhibition of SLC transporters by TKIs can be discarded.

The exact mechanism by which TKIs block activity of ENT1 remains to be determined. A possible implication of tyrosine kinase inhibition has to be considered, because such a mechanism is thought to contribute to SLC activity inhibition by TKIs. Indeed, inhibition of OATP1B1 by various TKIs involves repression of the Src kinase LYN-mediated OATP1B1 phosphorylation [29], whereas those of OCT1

![Fig. 3](image-url) Inhibition of ENT activity by lorlatinib in human lung A549 cells incubated with [3H]-uridine for 5 min at 37 °C, in the absence (control) or presence of A 0.1 µM NBMPR, 100 µM NBMPR, or 10 µM lorlatinib, or B various concentrations of lorlatinib. Intracellular accumulation of [3H]-uridine was next determined by scintillation counting and normalized to protein content. Data are expressed as A [3H]-uridine uptake or B % of ENT activity found in control cells, arbitrarily set at 100%, and are the means ± SD of three independent assays. A **p < 0.01 and ***p < 0.001, when compared to control cells. B Lorlatinib IC_{50} value is indicated on the top of the graph. ENT equilibrative nucleoside transporter, NBMPR nitrobenzylmercaptopurine riboside

![Fig. 4](image-url) Concentration-dependent inhibition of ENT2 activity by lorlatinib. HAP1 ENT1-KO cells were incubated with [3H]-uridine for 5 min at 37 °C, in the absence (control) or presence of various concentrations of lorlatinib. Intracellular uptake of [3H]-uridine was next determined by scintillation counting and normalized to protein content. Data are expressed as % of ENT2 activity found in control cells, arbitrarily set at 100%, and are the means ± SD of three independent assays. Lorlatinib IC_{50} value is indicated on the top of the graph. ENT equilibrative nucleoside transporter, KO knockout

![Fig. 5](image-url) Accumulation of lorlatinib in HAP1 wild-type, ENT1-KO and ENT2-KO cells. HAP1 wild-type, ENT1-KO, and ENT2-KO cells were incubated with 10 µM lorlatinib for 5 min at 37 °C, in the absence (control) or presence of 0.1 µM NBMPR or 100 µM NBMPR. Intracellular accumulation of lorlatinib was next determined by LC-MS/MS and normalized to protein content. Data are the means ± SD of four independent assays. NS not statistically significant. ENT equilibrative nucleoside transporter, KO knockout, NBMPR nitrobenzylmercaptopurine riboside
and OCT2 have been linked to inhibition of the kinase Yes1 [31, 32]. A major contribution of kinase activity inhibition to TKIs-mediated ENT1 inhibition is nevertheless rather unlikely, owing to (1) the time of incubation with TKIs (5 min), which may be shorter to induce changes in transporter phosphorylation status, and (2) the diverse nature of kinases targeted by TKIs inhibiting ENT1. Indeed, for example, lorlatinib blocks the kinase ROS1 and the anaplastic lymphoma kinase (ALK) [33], whereas ibrutinib represses activity of the Bruton’s tyrosine kinase (BTK) [34] and pacritinib those of JAK2 and Fms-like tyrosine kinase (Flt3) [35]. TKIs may consequently inhibit ENT1 through directly interacting with this transporter, as already reported for the ENT1 inhibitors, NBMPR and dilazep, for which binding sites on ENT1 have recently been characterized [36].

Various molecular descriptors have been associated with drug-mediated inhibition of SLCs acting as transporters. This notably concerns OATPs, OATs, OCTs, and MATEs, but not, or only poorly, ENT1 [27, 37–39]. In the present study, we have brought data about this nucleoside transporter, through identifying several physical/chemical parameters significantly correlated with ENT1 inhibition by TKIs. Among them, the atom-type E-state SssO and NssO exhibit the highest significant correlations; they correspond to electro-topological state indices, reflecting the combinations of electronic, topological, and valence state information [40]. The constitutional index, Mi, reflecting mean first ionization potential (scaled on carbon atom), as well as the functional group count, nArOR, corresponding to the number of ethers (aromatic), were also highly associated with TKI-mediated inhibition of ENT1 activity. Such parameters may consequently represent important descriptors to consider for establishing quantitative structure–activity relationship-based models, in order to predict ENT1 inhibition by drugs, notably TKIs.

Among five strong ENT1 inhibitors identified in the present study, only lorlatinib is predicted to in vivo inhibit ENT1, according to the criteria defined by the FDA guidance on DDIs [25]. This point may merit consideration in terms of possible DDIs and/or adverse effects. Indeed, even if ENT1 is presently not considered as a priority transporter according to the FDA [25], it has been postulated to be a transporter of emerging importance by the International Transporter Consortium, because its inhibition is susceptible to decrease the cellular uptake, and, in this way, the pharmacological effects, of anticancer nucleoside analogues, such as gemcitabine or cytarabine [15]. This has likely to be taken into account for lung cancer cells, which constitute pharmacological targets for lorlatinib [41]. Indeed, ENT1 activity is susceptible to be markedly inhibited by the TKI in these tumoral cells, as demonstrated in lung cancer A549 cells, which may result in a decreased ENT1-mediated accumulation of anticancer nucleoside analogues, and, possibly, in reduced anticancer activity of these drugs, if they are co-administrated with lorlatinib. Moreover, lorlatinib is predicted to also in vivo inhibit ENT2 activity, thus suggesting that this TKI may fully abrogate facilitated transport of nucleoside analogues into target cells and, by this way, may cause potential DDIs. Lorlatinib may also be suspected to reduce intestinal absorption of ENT1 substrates, through blocking their ENT1-related efflux at the basolateral membrane of enterocytes [42]; it may additionally inhibit ENT1-mediated drug transport at the hepatic and/or placental level. Nevertheless, ENT activity inhibition may have beneficial consequences, i.e., it may be hypothesized to contribute to the antiproliferative effects of lorlatinib, through reducing cellular uptake of nucleosides in cancer cells, in a kinase-independent manner. In addition, it may exert antithrombotic effects, through enhancing extracellular levels of the platelet repressor, adenosine, as already described for other ENT1 inhibitors [13]. Finally, it is noteworthy that lorlatinib is not a substrate for either ENT1 or ENT2, since (1) HAP1 wild-type, ENT1-KO, and ENT2-KO cells similarly accumulated the TKI, and (2) the ENT inhibitor, NBMPR, was without effect on lorlatinib uptake in these parental or ENT-KO cells, whenever it was used at 0.1 µM (inhibiting ENT1) or 100 µM (inhibiting both ENT1 and ENT2). ENT1 and ENT2 are therefore unlikely to play a role in lorlatinib pharmacokinetics or to contribute to its accumulation in target cells.

Inhibitory effects of TKIs towards ENT activity have been studied using functional knock-out cells, deficient in either ENT1 or ENT2 activity. The use of such cells for specifically investigating ENT1 or ENT2 activity therefore appears as a convenient alternative to that of transfected transporter-overexpressing cells, notably because native cells often display both ENT1 and ENT2 activities, even if, in the case of HAP1 wild-type cells, that of ENT1 is the main one. This conclusion is fully supported by the recent use of CRISPR/Cas9-generated HeLa cell variants, deficient in either ENT1 or ENT2, for characterizing ENT–drug interactions [14].

5 Conclusion

Various TKIs (12/24) used at 10 µM have been demonstrated to behave as moderate or strong inhibitors of ENT1, thus unambiguously adding ENT1 to the list of SLCs targeted by TKIs. As regards ALK-targeting lorlatinib, relevant clinical concentrations were even predicted to inhibit ENT1 activity in vivo, as well as that of ENT2. Lorlatinib was, however, not transported by ENT1 or ENT2, thus discarding any role of these nucleoside transporters in its cellular or general pharmacokinetics. Lorlatinib may thus appear as a potent inhibitor of ENT1, which likely merits attention if this TKI is co-administrated with known ENT1 substrates, like anticancer nucleoside analogues.
Declarations

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