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Differential *in vitro* interactions of the Janus kinase inhibitor ruxolitinib with human SLC drug transporters

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3 **Differential *in vitro* interactions of the Janus kinase inhibitor ruxolitinib with human SLC**
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5 **drug transporters**
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Abstract

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1. Interactions of the Janus kinase (JAK) inhibitor ruxolitinib with solute carriers (SLCs) remain incompletely characterized. The present study was therefore designed to investigate this issue.

2. The interactions of ruxolitinib with SLCs were analyzed using transporter-overexpressing HEK293 cells. Substrate accumulation was detected by spectrofluorimetry, LC-MS/MS spectrometry or scintillation counting.

3. Ruxolitinib was found to potently inhibit the activities of OAT3, OCT2, MATE1 and MATE2-K ($IC_{50} < 10 \mu\text{M}$). It blocked OAT1, OAT4, OATP1B1, OATP1B3, OATP2B1 and OCT3, but in a weaker manner ($IC_{50} > 10 \mu\text{M}$), whereas OCT1 was not impacted. No time-dependent inhibition was highlighted. When applying the US FDA criteria for transporters-related drug-drug interaction risk, OCT2 and MATE2-K, unlike MATE1 and OAT3, were predicted to be *in vivo* inhibited by ruxolitinib. Cellular uptake studies additionally indicated that ruxolitinib is a substrate for MATE1 and MATE2-K, but not for OAT3 and OCT2.

4. Ruxolitinib *in vitro* blocked activities of most of SLC transporters. Only OCT2 and MATE-2K may be however clinically inhibited by the JAK inhibitor, with the caution for OCT2 that *in vitro* inhibition data were generated with a FDA-non recommended fluorescent substrate. Ruxolitinib MATEs-mediated transport may additionally deserve attention for its possible pharmacological consequences in MATE-positive cells.

Key-words: ruxolitinib, SLC transporter, inhibition, substrate, OCT2, MATE

Introduction

The use of Janus Kinase (JAK) inhibitor drugs, termed JAKinibs, constitutes a recent therapeutic strategy targeting immune and inflammatory diseases, as well as myeloproliferative neoplasms (Schwartz *et al.* 2017, Vainchenker *et al.* 2018). One of these JAKinibs, *i.e.*, ruxolitinib exhibits potent anti-JAK1 and anti-JAK2 activity (Quintás-Cardama *et al.* 2011) and usually considered as a pan-JAK inhibitor (Febvre-James *et al.* 2018). It was initially approved by the US Food and Drug Administration (FDA) in 2011 for myelofibrosis and polycythemia vera treatment and more recently in 2019 for that of steroid-refractory acute graft-versus-host disease (Przepiorka *et al.* 2019). Ruxolitinib is additionally undergoing testing for its efficacy in treating the cytokine storm occurring during COVID-19 infection (Malavolta *et al.* 2020). Ruxolitinib acts as an ATP-competitive JAKinib and its 7H-pyrrolo[2,3-d]pyrimidine hinge binding motif is thought to be required for its activity (Gehring *et al.* 2014).

Membrane drug transporters are considered to play a key role in pharmacokinetics of xenobiotics and endogenous compounds. They notably contribute to the role of barrier of some tissues like gut or brain capillary endothelium, especially thanks to ATP Binding Cassette (ABC) transporters, acting as efflux pumps. Barrier and detoxifying organs also contain Solute Carrier (SLC) transporters, allowing mainly influx transport of xenobiotics, with only few of them, like multidrug and toxin extrusion proteins (MATEs/*SLC47A*), acting *in vivo* as efflux transporters (Kusakizako *et al.* 2019). Both ABC efflux and SLC influx transporters can be responsible of drug-drug interactions (DDIs), through inhibition (by perpetrators) of the transport of drugs (considered as victims) (Gessner *et al.* 2019). Their studies are now required during the development of a new chemical entity (NCE) by most of drug agencies (The International Transporter Consortium 2010, Kusakizako *et al.* 2019). The list of these transporters which have to be regulatory considered is growing, with the addition of emergent transporters like MATEs (U.S. Department of Health and Human Services, FDA 2017).

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3 While the ruxolitinib metabolism pathway is rather well-characterized (ruxolitinib is
4 mainly metabolized by cytochrome P450 3A4 (CYP3A4) and to a lower extent by CYP2C9
5 (Ogama *et al.* 2013, Shi *et al.* 2012, Agarwal *et al.* 2013)), its interaction with transporters
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7 (Ogama *et al.* 2013, Shi *et al.* 2012, Agarwal *et al.* 2013)), its interaction with transporters
8 remains to date less documented and some conflicting results about this issue have been
9
10 reported. Thus, the ABC transporters P-glycoprotein (P-gp/*ABCB1*) and breast cancer
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12 resistance protein (BCRP/*ABCG2*) have initially been reported to not interact with ruxolitinib
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14 (Agarwal *et al.* 2013), but other data do not agree with this conclusion (Shi *et al.* 2015, Ebert
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16 *et al.* 2016, Gay *et al.* 2017). In the same way, SLC transporters, such as organic anion-
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18 transporting polypeptides (OATPs/*SLCOs*) and organic cation/organic anion transporters
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20 (OCTs and OATs/*SLC22A* family), were initially thought to not *in vivo* interact with ruxolitinib
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22 (Agarwal *et al.* 2013), but moderate *in vitro* inhibitions of OATP1B1 (*SLCO1B1*) or of OCT2
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24 (*SLC22A2*) have been recently described when using ruxolitinib at 10 μ M (Hu *et al.* 2014,
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26 Sprowl *et al.* 2016), a concentration closed to those reached *in vivo* (Shi *et al.* 2011). Besides,
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28 the putative inhibitions by the JAKinib of MATEs and other emergent transporters, such as
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30 OCT3, sharing many substrates with OCT1 and OCT2 (Koepsell 2020), and OAT4, which may
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32 be implicated in renal reabsorption of drugs (Burckhardt 2012), have not yet been investigated,
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34 according to the best of our knowledge. The possible transport of ruxolitinib by SLCs remains
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36 also poorly characterized (Alim *et al.* 2020). Therefore, even if some data about interactions of
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38 ruxolitinib with drug transporters are freely available online (Center for Drug Evaluation and
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40 Research 2011), additional studies about this topic are likely needed to confirm and extend
41
42 previous results, notably with respect to emergent SLC transporters, including MATEs. Such
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44 studies may use fluorescent reference substrates for some transporter inhibition assays, which
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46 does not requires the use of high-cost and specific analytical methods like scintillation counting
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48 or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Fardel *et al.*
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50 2015). The present work was therefore designed to evaluate ruxolitinib interactions with main
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3 SLC drug transporters, including emergent ones, implicated in pharmacokinetics, using partly
4 fluorescent substrates for some of the transporter inhibition assays, and to compare results with
5 those previously obtained. Our data highlighted potential clinical inhibition of OCT2 by
6 ruxolitinib and also transport of the JAKinib by MATEs.
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14 **Materials and Methods**

15 *Chemicals*

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18 4',5'-dibromofluorescein (DBF), 2',7'-dichlorofluorescein (DCF), 6-carboxyfluorescein (6-CF),
19 amitriptyline, corticosterone, probenecid, rifamycin SV, sulfobromophthalein (BSP) and
20 tetraethylammonium (TEA) were from Sigma-Aldrich (Saint Quentin Fallavier, France),
21 4-(4-(dimethylamino)styryl)-N-methylpyridiniumiodide (4-Di-ASP) from Thermo Fisher
22 Scientific (Waltham, MA) and 8-fluorescein-cAMP (8-FcA) from BioLog Life Science
23 Institute (Bremen, Germany). [6,7-³H(N)]- estrone-3-sulfate (E3S) (specific activity of 54
24 Ci/mmol) was provided by PerkinElmer (Boston, MA). Stocked solutions of chemicals were
25 commonly prepared in dimethyl sulfoxide (DMSO); final concentrations of solvent in transport
26 assay medium did not exceed 0.1 % (vol/vol).
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43 *Cell culture*

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46 Human embryonic kidney cell line (HEK293 cells) stably overexpressing OAT1 (HEK-OAT1
47 cells), OAT3 (HEK-OAT3 cells), OAT4 (HEK-OAT4 cells), OATP1B1 (HEK-OATP1B1),
48 OATP1B3 (HEK-OATP1B3), OATP2B1 (HEK-OATP2B1 cells), OCT1 (HEK-OCT1 cells),
49 OCT2 (HEK-OCT2 cells), OCT3 (HEK-OCT3 cells), MATE1 (HEK-MATE1 cells) or
50 MATE2-K (HEK-MATE2-K cells), as well as control HEK293-MOCK cells were cultured in
51 DMEM medium (Thermo Fisher Scientific, Villebon sur Yvette, France) containing 4.5 g/L D-
52 glucose and supplemented with 10% fetal calf serum (vol/vol), 1 µg/mL bovine insulin (Sigma-
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3 Aldrich), 1% non-essential amino acid solution (Thermo Fisher Scientific), 20 UI/mL penicillin
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5 and 20 µg/mL streptomycin. G418 (800 µg/mL) was added for the culture of HEK-OCT3 cells,
6
7 as a selection agent. All these SLC transporter-overexpressing HEK293 cells have previously
8
9 been described and validated (Sayyed *et al.* 2019, Nies *et al.* 2011, Chedik *et al.* 2017).

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12 For functional transport assays, cells were plated in 48-wells or 96-wells plates coated
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14 with poly-D-lysine to enhance cell adhesion, at a density of 4×10^4 or 1×10^4 cells/well,
15
16 respectively, and were used when reaching confluence (usually 5 days after seeding).
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20 21 ***Transporter assays***

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24 Transporter assays were performed as previously described (Chedik *et al.* 2017). Briefly, for
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26 *cis*-inhibition studies, cells were incubated for 5 min at 37°C in a transport assay buffer
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28 containing a reference substrate of the considered transporter, in the presence or absence of a
29
30 reference inhibitor or ruxolitinib. The 5 min incubation time was already demonstrated to be in
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32 the linear uptake range for transporters and is commonly used for transport assays with HEK-
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34 293 cell lines (Le Vée *et al.* 2019, Jouan *et al.* 2014, Fardel *et al.* 2015, Müller *et al.* 2013). For
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36 the determination of potential time-dependent inhibition, ruxolitinib pre-incubation was
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38 performed for 30 min as recommended by the 2017 US FDA guidance entitled “In Vitro
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40 Metabolism- and Transporter- Mediated Drug-Drug Interaction Studies Guidance for Industry”
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42 (U.S. Department of Health and Human Services, FDA 2017) . The assay buffer consisted in
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44 136 mM NaCl, 5.3 mM KCl, 1.1mM KH₂PO₄, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 10 mM HEPES
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46 and 11 mM D-glucose, adjusted to pH=7.4 (8.4 for organic cation/proton exchangers MATEs).
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49 The nature and concentrations of substrates and inhibitors used for each investigated SLC
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51 transporter have previously been well documented (Le Vée *et al.* 2019) and are summarized in
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53 Table 1. Cells were next washed twice with ice-cold phosphate-buffered saline (PBS), and lysed
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55 in distilled water. Intracellular accumulation of reference substrates was finally determined by
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scintillation counting using a Tri-Carb© 2910TR analyser (PerkinElmer) for [³H]-E3S or by spectrofluorimetry using a SpectraMax Gemini SX spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) for 6-CF, DBF, DCF, 8-FcA and 4-Di-ASP; preliminary experiments indicated that ruxolitinib did not decrease transporter probes-related fluorescence in HEK-MOCK cells (Figure S1), thus supporting the absence of major analytical interference of the JAKinib with the fluorescent dyes used as substrates in a cellular context. 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, Les Ulis, France) and coupled to a tandem mass spectrometry TSQ Vantage (Thermo Fisher Scientific, Villebon sur Yvette, France), fitted with an electrospray ionization source (ESI+), was additionally used for analyzing unlabeled TEA; monitored ion transitions were at 130.2 > 86.1 m/z. Data were then normalized to total protein content, determined by the Bradford's method (Bradford 1976). Data were routinely expressed as percentages of substrate accumulation found in the absence of ruxolitinib or reference inhibitors according to the following equation (A):

$$\% \text{ Substrate accumulation} = \frac{([\text{Substrate}_{\text{ruxolitinib or inhibitor}}] \times 100)}{([\text{Substrate}_{\text{control}}])} \quad (\text{A})$$

with $[\text{Substrate}_{\text{ruxolitinib or inhibitor}}]$ = cellular concentration of reference substrate in the presence of a defined concentration of ruxolitinib or reference inhibitor and $[\text{Substrate}_{\text{control}}]$ = cellular concentration of reference substrate in cells not exposed to ruxolitinib or reference inhibitor.

Some data were also expressed as % of residual SLC transporter activity according to the following equation (B):

$$\% \text{ Transporter activity} = \frac{([\text{Substrate}_{\text{ruxolitinib}}] - [\text{Substrate}_{\text{ref inhibitor}}]) \times 100}{([\text{Substrate}_{\text{control}}] - [\text{Substrate}_{\text{ref inhibitor}}])} \quad (\text{B})$$

with $[\text{Substrate}_{\text{ruxolitinib}}]$ = cellular concentration of reference substrate in the presence of a defined concentration of ruxolitinib, $[\text{Substrate}_{\text{ref inhibitor}}]$ = cellular concentration of reference substrate in the presence of a defined concentration of reference inhibitor and $[\text{Substrate}_{\text{control}}]$

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3 = cellular concentration of reference substrate in cells not exposed to ruxolitinib or reference
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5 inhibitor. [Table 1 near here]
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8 9 10 ***Determination of half maximal inhibitory concentrations (IC₅₀)***

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12 IC₅₀ values were determined in most cases if ruxolitinib inhibited more than 50% of a
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14 transporter activity when incubated at 10 μM. For this, the effects of various ruxolitinib
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16 concentrations (from 0.01 to 100 μM) towards activity of the incriminated transporter were
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18 determined as described above. The time-dependent inhibition, *i.e.*, the effects of 30 min pre-
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20 incubation with ruxolitinib, was also determined towards these activities according to FDA
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22 recommendations to determine a potential IC₅₀ shift (U.S. Department of Health and Human
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24 Services, FDA 2020). IC₅₀ values were next determined using Prism 5.0 software (GraphPad
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26 Software, La Jolla, CA, USA) through nonlinear regression based on the following four-
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28 parameter logistic equation (C):
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$$32 \quad A = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{LogIC}_{50} - [I]) \times \text{Hill slope}}} \quad (C)$$

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34 where A is the percentage of transporter activity for a given concentration of ruxolitinib, [I] is
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36 the ruxolitinib concentration in the medium, Hill slope is a coefficient describing the steepness
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38 of the curve and Bottom and Top are the highest and lowest residual activity, respectively
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40 (usually fixed at 0 and 100, respectively).
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46 47 ***Ruxolitinib accumulation assays***

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49 HEK-MOCK, HEK-OAT3, HEK-MATE1, HEK-MATE2-K and HEK-OCT2 cells were
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51 incubated with 100 μM ruxolitinib, in the absence or presence of reference transporter inhibitors
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53 (2 mM probenecid, 100 μM verapamil or 100 μM amitriptyline for OAT3, MATE or OCT2
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55 inhibition, respectively), for 5 min at 37°C, in the transport assay medium already described
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57 above. The use of the 100 μM concentration of ruxolitinib was selected for favouring analytical
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determination of intracellular levels of the JAKinib. Cells were then washed twice in ice-cold PBS, lysed in distilled water and acetonitrile-based extraction of cell lysates was performed. Ruxolitinib quantification was next performed through LC-MS/MS using the system described above; ruxolitinib monitored ion transitions were at 307.1 > 186.1 m/z. Data were finally normalized to protein content determined by the Bradford's method (Bradford 1976).

Prediction of in vivo modulation of SLC transporter activity by ruxolitinib

In vivo modulation of transporter activities by ruxolotinib was evaluated from *in vitro* data using the criteria defined by the US FDA guidance on *in vitro* drug interaction studies (U.S. Department of Health and Human Services, FDA) Briefly, OAT1, OAT3, OCT2, MATE1 or MATE2-K can be *in vivo* inhibited by ruxolitinib if:

$$I_{\max, u} / IC_{50} \geq 0.1 \quad (D)$$

with $I_{\max, u}$ = maximum unbound plasma concentration of ruxolitinib and IC_{50} = half maximal inhibitory concentration. The equation (D) was also retained for evaluating *in vivo* inhibition of OCT3 and OAT4.

For OATP1B1 and OATP1B3, expressed at the sinusoidal membrane of hepatocytes, an *in vivo* inhibition can be considered according to the US FDA if:

$$R = 1 + ((f_{u,p} \times I_{in,max}) / IC_{50}) \geq 1.1 \quad (E)$$

with $f_{u,p}$ = unbound fraction in plasma, IC_{50} = half maximal inhibitory concentration and $I_{in,max}$ = estimated maximum plasma inhibitor concentration at the inlet to the liver, calculated as:

$$I_{in,max} = I_{\max} + (F_a \times F_g \times k_a \times Dose) / Q_h / R_B \quad (F)$$

with I_{\max} = maximum plasma concentration of ruxolitinib, F_a = fraction absorbed, F_g = intestinal availability, k_a = absorption rate constant, Q_h = hepatic blood flow rate and R_B = blood-to-plasma concentration ratio (If unknown, $F_a = 1$, $F_g = 1$ and $k_a = 0.1/\text{min}$ can be used as a worst-case estimate).

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3 The equation (E) was also used for evaluating *in vivo* inhibition of OATP2B1 and OCT1,
4 expressed notably at the sinusoidal membrane of hepatocytes (Le Vée *et al.* 2015).
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10 ***Statistical analysis***

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12 Experimental data were routinely expressed as means \pm standard deviations (SD). They were
13 statistically analyzed through analysis of variance (ANOVA) followed by the Dunnett's or the
14 Tukey's post-hoc test. IC₅₀ values determined with and without pre-incubation with ruxolitinib
15 were compared with the F-test. The criterion of significance was $p < 0.05$.
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24 **Results**

25 ***Interactions of ruxolitinib with SLC transporters of anionic drugs***

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27 Ruxolitinib was first tested towards OAT activities, using HEK-OAT cells. Such cells displayed
28 fully functional OAT activities, as demonstrated by the marked inhibition of 6-CF accumulation
29 in HEK-OAT1 and HEK-OAT3 cells, as well as that of E3S in HEK-OAT4 cells, by the
30 reference OAT inhibitor probenecid (Figure 1A). Ruxolitinib was found to inhibit OAT
31 activities in a concentration-dependent manner (Figure 1A). While the JAK inhibitor used at
32 100 μ M significantly reduced accumulation of 6-CF in HEK-OAT1 and HEK-OAT3 cells and
33 that of E3S in HEK-OAT4 cells, the lower concentration of 10 μ M failed to reduce E3S
34 accumulation in HEK-OAT4 cells and only modestly, but significantly, decreased that of 6-CF
35 in HEK-OAT1 cells by 36% (Figure 1A). This indicated that IC₅₀ values of ruxolitinib towards
36 OAT1 and OAT4 activities are $> 10 \mu$ M (Table 2). By contrast, ruxolitinib used at 10 μ M
37 reduced 6-CF accumulation in HEK-OAT3 cells by 67% (Figure 1A). IC₅₀ values towards
38 OAT3 activity were estimated at $3.1 \pm 1.4 \mu$ M (without pre-incubation) and $2.9 + 1.1 \mu$ M (with
39 a 30 min-pre-incubation) (Figure 1B), indicating no time-dependent inhibition ($p > 0.05$).
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60 Similar accumulation of ruxolitinib in HEK-OAT3 and HEK-MOCK cells was further

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3 demonstrated by LC-MS/MS analysis, without any significant effect of the OAT inhibitor
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5 probenecid (Figure 1C). This supports a lack of transport of ruxolitinib by OAT3. [Figure 1
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7 and Table 2 near here]

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10 The effects of ruxolitinib towards SLC transporter activities were next characterized in
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12 HEK-OATP cells. OATPs were fully active in such cells, as demonstrated by the significant
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14 inhibitions of the cellular accumulations of reference OATP substrates (DCF, 8-FcA and DBF
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16 for OATP1B1, OATP1B3 and OATP2B1, respectively) by the OATP inhibitors BSP and
17
18 rifamycin SV (Figure 2A). Ruxolitinib used at 100 μM markedly inhibited accumulation of the
19
20 reference OATP substrates in HEK-OATP1B1, HEK-OATP1B3 and HEK-OATP2B1 cells
21
22 (Figure 2A); the concentration of 10 μM however failed to reduce substrate accumulation in
23
24 HEK-OATP1B3 and HEK-OATP2B1 cells and only weakly, but significantly, decreased it in
25
26 HEK-OATP1B1 cells by 32%. This indicated that IC_{50} values of ruxolitinib towards activities
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28 of OATP1B1, OATP1B3 and OATP2B1 are $> 10 \mu\text{M}$ (Table 2). For OATP1B1, IC_{50} value was
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30 determined at $14.2 \pm 1.1 \mu\text{M}$ without pre-incubation with ruxolitinib (Figure 2B); with a 30 min
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32 pre-incubation, IC_{50} value ($12.2 \pm 1.1 \mu\text{M}$) was not statistically different [Figure 2 near here]
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40 ***Interactions of ruxolitinib with SLC transporters of cationic drugs***

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42 The effects of ruxolitinib towards OCT activities were determined in HEK-OCT cells,
43
44 displaying functional OCT activities, as demonstrated by the inhibition of 4-Di-ASP
45
46 accumulation by the reference OCT inhibitors amitriptyline and corticosterone (Figure 3A).
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48 Ruxolitinib used at 10 or 100 μM failed to alter 4-Di-ASP accumulation in HEK-OCT1 cells
49
50 (Figure 3A), indicating that it was ineffective towards OCT1 ($\text{IC}_{50} > 100 \mu\text{M}$) (Table 2). It
51
52 significantly reduced 4-Di-ASP uptake in HEK-OCT3 cells, but only when used at 100 μM
53
54 (Figure 3A), therefore supporting a ruxolitinib $\text{IC}_{50} > 10 \mu\text{M}$ towards OCT3 activity (Table 2).
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57 The JAK inhibitor more markedly inhibited 4-Di-ASP accumulation in HEK-OCT2 cells, with
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3 a near full inhibition of the cellular accumulation of the reference OCT substrate in response to
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5 10 and 100 μM ruxolitinib (Figure 3A). Ruxolitinib IC_{50} values towards OCT2 activity were
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7 further determined to be $1.2 \pm 1.3 \mu\text{M}$ (without pre-incubation) and $1.7 \pm 1.2 \mu\text{M}$ (with pre-
8
9 incubation) (Figure 3B), indicating the absence of time-dependent inhibition ($p > 0.05$). Similar
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11 accumulation of ruxolitinib in HEK-OCT2 and HEK-MOCK cells was finally demonstrated by
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13 LC-MS/MS analysis, without any significant effect of the OCT inhibitor amitriptyline (Figure
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15 3C). This most likely indicates that ruxolitinib is not transported by OCT2. [Figure 3 near here]

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19 Ruxolitinib inhibitory properties were next tested in HEK-MATE1 and HEK-MATE2-K
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21 cells. These HEK-MATE cells exhibited functional MATE activities, as assessed by the
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23 verapamil-inhibited accumulation of the reference MATE substrate TEA (Figure 4A).
24
25 Ruxolitinib used at 10 or 100 μM was found to markedly inhibit TEA accumulation in HEK-
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27 MATE1 and HEK-MATE2-K cells, by more than 60 % in response to the 10 μM concentration
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29 (Figure 4A). IC_{50} values towards MATE1 and MATE2-K activities in the absence of pre-
30
31 incubation were next estimated to $2.5 \pm 1.8 \mu\text{M}$ and $1.1 \pm 1.2 \mu\text{M}$, respectively (Figure 4B),
32
33 without any significant impact of pre-incubation step (IC_{50} values after a 30 min-preincubation
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35 were 2.3 ± 1.6 and $1.0 \pm 1.14 \mu\text{M}$ for MATE1 and MATE2-K, respectively). HEK-MATE1 and
36
37 HEK-MATE2-K cells were then demonstrated to display enhanced cellular accumulation of
38
39 ruxolitinib when compared to HEK-MOCK cells (by 3.2- and 3.8-fold factors for HEK-MATE1
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41 and HEK-MATE2-K cells, respectively) (Figure 4C). Co-incubation of verapamil suppressed
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43 this increased ruxolitinib accumulation in HEK-MATE1 and HEK-MATE2-K cells, without
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45 affecting ruxolitinib levels in HEK-MOCK cells (Figure 4C). Such data support a transport of
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47 ruxolitinib by MATE1 and MATE2-K. [Figure 4 near here]

52 53 54 55 56 ***Prediction of in vivo inhibition of SLC transporters by ruxolitinib***

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Potential *in vivo* inhibition of SLC transporter activities by ruxolitinib was predicted using the criteria defined by the US FDA for transporters-related DDIs. We obtained a maximal plasma concentration (I_{\max}) of 7.2 μM for ruxolitinib, whose unbound fraction is 0.03, according to US FDA data (Center for Drug Evaluation and Research 2011). This resulted in a $I_{\max,u}$ value of 0.216 μM for ruxolitinib. With such an $I_{\max,u}$ and the IC_{50} values determined in the present study using reference (like TEA) or fluorescent substrates, the ratio $I_{\max,u}/IC_{50}$ reached the threshold of 0.1 for only OCT2 and MATE2-K, indicating that these transporters were susceptible to be *in vivo* blocked by ruxolitinib, unlike OCT3, OATs and MATE1 (Table 2). For OATPs and OCT1, we calculated an $I_{in,max} = 17.7 \mu\text{M}$ (with $I_{\max} = 7.2 \mu\text{M}$ at Dose = 200 mg; $F_a = 0.99$, $F_g = 0.87$, $k_a = 2.02 \text{ h}^{-1}$ and $R_B = 1.2$ according to Umehara et al. (Umehara *et al.* 2019)). This led to R values lower than 1.1 for OCT1, OATP1B1, OATP1B3 and OATP2B1, discarding any *in vivo* inhibition of OCT1 or OATPs by ruxolitinib (Table 2).

Comparison with FDA data for in vitro SLC transporter inhibition by ruxolitinib

We finally compared data from our present study to those from the FDA report on ruxolitinib (Center for Drug Evaluation and Research 2011), with respect to ruxolitinib-mediated *in vitro* inhibition of OATs, OATPs and OCTs. As shown in Table 3, 4/6 transporters, *i.e.*, OAT3, OATP1B1, OATP1B3 and OCT2, were found to be inhibited by ruxolitinib in the two studies. According to IC_{50} values, OCT2 was however almost 10-fold more sensitive to ruxolitinib in our study (based on 4-Di-ASP transport) than in that reported by the FDA (based on 1-methyl-4-phenylpyridinium transport). Divergent *in vitro* effects of ruxolitinib were reported for 2/6 transporters: (i) OAT1, which is rather moderately inhibited by ruxolitinib in our study, was not sensitive to ruxolitinib in the FDA report, knowing however that the maximal tested concentration in the FDA report was 37.5 μM (versus 100 μM in our study) and (ii) OCT1, inhibited by ruxolitinib according to the FDA (with an $IC_{50} < 10 \mu\text{M}$), was not impaired by

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3 ruxolitinib, even at the high concentration of 100 μM , in our present work (Table 3). [Table 3
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5 near here] It is however noteworthy that the OAT1 and OCT1 substrates used in our study
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7 (fluorescent substrates like 6-CF and 4-Di-ASP) were different from those used in the FDA
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9 report (aminohippuric acid and 1-methyl-4-phenylpyridinium).
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14 **Discussion**

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16 The present study demonstrates that the JAK inhibitor ruxolitinib differentially *in vitro* interacts
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18 with the main SLC drug transporters. If ruxolitinib used at the high concentration of 100 μM
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20 was able to inhibit most of SLC transporters usually implicated in drug pharmacokinetics (at
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22 absorption and/or distribution and/or elimination steps), such as OAT1, OAT3, OATP1B1,
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24 OATP1B3, OATP2B1, OCT2, MATE1 and MATE2-K, only activities of OAT3, OCT2,
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26 MATE1 and MATE2-K were suppressed with ruxolitinib $\text{IC}_{50} < 10 \mu\text{M}$. In addition, when
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28 applying the criteria of FDA for clinical prediction of DDI, only OCT2 and MATE2-K are
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30 susceptible to be *in vivo* blocked by ruxolitinib. This suggests that administration of drugs
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32 substrates for OCT2 and/or MATE2-K and subjected to renal secretion, such as metformin or
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34 cimetidine (Wang *et al.* 2008), may require special attention in terms of DDI for patients already
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36 treated by ruxolitinib. By contrast, for other SLC transporters, any putative DDIs due to
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38 inhibition of transporter activity by ruxolitinib may likely be discarded.
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45 Comparison of the data found in the present study with those previously reported by the
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47 FDA reveals some similarities, notably for OAT3, OATP1B1 and OATP1B3 (Table 3). OCT2
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49 was also found to be inhibited by ruxolitinib in the two studies, even if this transporter was 10-
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51 fold more sensitive to ruxolitinib in our present study. In the same way, 10 μM ruxolitinib
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53 inhibited OCT2-mediated transport by approximately 80% in the present work (Figure 3A), but
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55 by only 60% in the work of Sprowl *et al.* (2016). Such a higher *in vitro* sensitivity likely explains
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57 why *in vivo* inhibition of OCT2 by ruxolitinib was predicted to occur in the present study, but
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3 not in the FDA report. It is noteworthy that the substrate probe used in our functional OCT2
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5 assay (4-Di-ASP) differs from that used in the study reported by FDA (1-methyl-4-
6
7 phenylpyridinium) (Table 3) and in that of Sprowl et al (2016) (TEA). This is likely to account
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9 for the differential OCT2 sensitivity to the JAK inhibitor, because the profile of OCT2
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11 inhibition is well-known to be substrate-dependent (Belzer *et al.* 2013); in particular, OCT2-
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13 mediated 1-methyl-4-phenylpyridinium transport (used in the study of the FDA report) has been
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15 demonstrated to be less sensitive to inhibition than that of 4-Di-ASP (used in the present study)
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17 (Sandoval *et al.* 2018). This substrate-related dependence of transporter inhibition, also
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19 described for other SLC transporters like OATP1B1 (Izumi *et al.* 2013) and MATE1 (Martínez-
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21 Guerrero and Wright 2013) and for ABC transporters (Pedersen *et al.* 2017), can be
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23 hypothesized to also explain the lack of OCT1 inhibition by ruxolitinib in our study, whereas,
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25 by contrast, OCT1 activity was found to be suppressed by the JAK inhibitor in the study
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27 reported by FDA; indeed, different OCT1 substrates were used in the two studies (Table 3).
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29 The use of different substrates also likely contributes to the fact that OAT1 was inhibited by
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31 ruxolitinib in the present study, but not in the FDA report. Overall, such data highlight the
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33 importance of the substrate nature in drug transporter inhibition analyses, even if the exact
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35 clinical consequences of such substrate-dependent transporter inhibition profiles remain to be
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37 established. This supports the recent recommendation of the FDA that the inhibition constant
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39 of a tested drug should be determined with a probe substrate that may also be used in later
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41 clinical studies or, alternatively, that usually generates a lower IC_{50} for known inhibitors, to
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43 avoid underestimating the interaction potential of the investigational drug (U.S. Department of
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45 Health and Human Services, FDA 2020). In this context, fluorescent dyes may be particularly
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47 interesting to consider, because their SLC-mediated transport may be highly sensitive to
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49 inhibitors, as previously reported for OCT2-related transport of 4-DiASP (Sandoval *et al.*
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3 2018). Additionally, inter-laboratory variability may also contribute to variability in potency
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5 (IC₅₀) of transporter inhibitors, as already underlined for P-gp inhibition (Bentz *et al.* 2013).
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8 The capacity of ruxolitinib to inhibit the transporters MATE1, MATE2-K, OATP2B1,
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10 OAT4 and OCT3, was investigated for the first time in the present study. In this context, it is
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12 noteworthy that MATE1 and MATE2-K have to be regulatory studied during the preclinical
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14 development of new drugs (Zamek-Gliszczyński *et al.* 2018), whereas OATP2B1 is considered
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16 as a transporter of emerging clinical importance, which may therefore also be evaluated, notably
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18 in a retrospective manner (Zamek-Gliszczyński *et al.* 2018). By contrast, there is presently no
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20 regulatory recommendation for the study of OCT3 and OAT4, probably because such
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22 transporters are presently not thought to play a major role in pharmacokinetics, even if they are
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24 expressed in organs playing a main role in drug disposition such as the liver, *i.e.*, OCT3 is
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26 present at the sinusoidal membrane of hepatocytes (Nies *et al.* 2009), and the kidney, *i.e.*, OAT4
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28 is present at the apical site of proximal tubular cells (Ekaratanawong *et al.* 2004). Ruxolitinib
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30 was found to moderately *in vitro* block OAT4 and OCT3 (inhibition in response to 100 µM
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32 ruxolitinib) and more markedly MATE1 and MATE2-K (inhibition in response to 10 µM
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34 ruxolitinib) and more markedly MATE1 and MATE2-K (inhibition in response to 10 µM
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36 ruxolitinib), but none of these transporters was predicted to be *in vivo* inhibited by the JAK
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38 inhibitor, with a potential exception for MATE2-K, as its R-value is higher than 0.1.
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42 Among transporters which were rather markedly inhibited by ruxolitinib *in vitro*, *i.e.*,
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44 OAT3, OCT2, MATE1 and MATE2-K, only MATE1 and MATE2-K were found to transport
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46 the JAK inhibitor, as demonstrated by ruxolitinib accumulation assays. A contribution of OAT3
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48 and OCT2, basically expressed at the basolateral membrane of proximal tubular cells and
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50 involved in renal secretion of drugs (Morrissey *et al.* 2013), to ruxolitinib pharmacokinetics can
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52 therefore be discarded. By contrast, MATEs, expressed at the apical membrane of hepatocytes
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54 (MATE1) and renal proximal tubular cells (MATE1 and MATE2-K) (Nies *et al.* 2016), may
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56 participate to hepatic and renal elimination of the JAK inhibitor. This putative excretion of
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3 ruxolitinib via MATEs should however remain a minor way of elimination, as most of the drug
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5 is eliminated by the metabolic pathway (Ogama *et al.* 2013, Agarwal *et al.* 2013) and its renal
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7 secretion is very low (Shilling *et al.* 2010), thus reducing the risk of DDI due to *in vivo*
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9 inhibition of MATEs-mediated transport of the JAK inhibitor. Nevertheless, it is noteworthy
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11 that MATE1 is expressed by myeloid cells (Harrach *et al.* 2016), including macrophages (Berg
12
13 *et al.* 2018), which can constitute specific targets for ruxolitinib (Febvre-James *et al.* 2018). In
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15 such cells, MATE1 may therefore be hypothesized to function as an uptake transporter for
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17 ruxolitinib, as already demonstrated for the tyrosine kinase inhibitor imatinib (Harrach *et al.*
18
19 2016). By this way, MATE1 may control the intracellular effectiveness of ruxolitinib. Further
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21 studies, investigating notably ruxolitinib accumulation in blood and bone marrow cells, are
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23 however required to validate this hypothesis.
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29 Direct interaction with transporter activity is not the only way by which ruxolitinib may
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31 cause DDIs. Indeed, the JAK inhibitor has been reported to fully suppress interleukin (IL-6)-
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33 mediated repression of drug transporters such as OATP1B1 and OCT1, as well as that of
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35 cytochrome P-450 3A4, through antagonizing the JAK-dependent signaling cascade of IL-6
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37 (Febvre-James *et al.* 2017). Ruxolitinib may consequently restore hepatic detoxification
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39 capacity for patients suffering from inflammatory diseases, which may in turn cause DDIs, as
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41 already described for other anti-IL-6 agents, like tocilizumab (Schmitt *et al.* 2011). Such effects
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43 are likely not restricted to IL-6, because the JAK inhibitor also prevents drug detoxifying
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45 repression triggered by IL-22 in human hepatocytes (Le Vée *et al.* 2020). Besides, ruxolitinib
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47 is likely to exert a global anti-inflammatory effect (Roskoski 2016), notably through
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49 suppressing hepatic up-regulation of acute-phase proteins like C-reactive protein (CRP)
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51 (Febvre-James *et al.* 2020) and production of inflammatory cytokines in macrophages (Febvre-
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53 James *et al.* 2018). Such a general inflammation suppression may contribute to prevent the well-
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3 established deleterious consequences of inflammatory states with respect to drug transporter
4 expression (Aitken *et al.* 2006).
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10 **Conclusion**

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12 To conclude, ruxolitinib was shown to differentially interact with SLC drug transporters *in*
13 *vitro*. The anionic drug transporter OAT3 and the cationic drug transporters OCT2, MATE1
14 and MATE2-K were notably found to be inhibited by relative low concentrations of ruxolitinib
15 (IC₅₀ < 10 μM), but only OCT2 and MATE2-K were predicted to be *in vivo* blocked by the JAK
16 inhibitor. MATE1 and MATE2-K, unlike OAT3 and OCT2, were additionally demonstrated to
17 transport ruxolitinib, which may have to deserve attention, notably with respect to ruxolitinib
18 effectiveness in MATE1-positive cells.
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30 **Conflict of interest**

31 The authors have no conflict of interest to declare.
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Figure 1. Interactions of ruxolitinib with OATs.

(A) Accumulation of reference substrates (6-CF for OAT1 and OAT3, E3S for OAT4) was determined in HEK-OAT cells incubated in the absence (control) or presence of ruxolitinib (10 or 100 μM) or of the reference OAT inhibitor probenecid (Prob) (2 mM). Data are expressed as % of substrate accumulation found in control cells, arbitrarily set at 100%, and are the means \pm SD of three independent assays, each being performed in triplicate. **, $p < 0.01$ and ***, $p < 0.001$, when compared to control cells. (B) Effects of various concentrations of ruxolitinib on 6-CF-related OAT3 activity were analyzed in HEK-OAT3 cells with or without pre-incubation. Data are expressed as % of OAT3 activity in control cells not exposed to ruxolitinib, arbitrarily set at 100%; they are the means \pm SD of three independent assays. IC_{50} values are indicated at the top of the graph. (C) Accumulation of ruxolitinib in HEK-OAT3 and HEK-MOCK cells exposed or not to 2 mM probenecid was determined by LC-MS/MS. Data are the means \pm SD of three independent assays, each being performed in triplicate. NS, not statistically significant.

Figure 2. Effects of ruxolitinib on OATP activities.

(A) Accumulation of reference substrates (DCF for OATP1B1, 8-FcA for OATP1B3, DBF for OATP2B1) was determined in HEK-OATP cells incubated in the absence (control) or presence of ruxolitinib (10 or 100 μM) or of the reference OATP inhibitors BSP (100 μM) or rifamycin SV (Rif SV) (100 μM). Data are expressed as % of substrate accumulation found in control cells, arbitrarily set at 100%, and are the means \pm SD of three independent assays, each being performed in triplicate. *, $p < 0.05$ and ***, $p < 0.001$, when compared to control cells. (B) Effects of various concentrations of ruxolitinib on DCF-related OATP1B1 activity were analyzed in HEK-OATP1B1 cells with or without pre-incubation with ruxolitinib. Data are

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3 expressed as % of OATP1B1 activity in control cells not exposed to ruxolitinib, arbitrarily set
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5 at 100%; they are the means \pm SD of three independent assays. IC₅₀ values are indicated at the
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7 top of the graph.
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12 **Figure 3.** Interactions of ruxolitinib with OCTs.

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14 (A) Accumulation of the reference OCT substrate 4-Di-ASP was determined in HEK-OCT cells
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16 incubated in the absence (control) or presence of ruxolitinib (10 or 100 μ M) or of the reference
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18 OCT inhibitors amitriptyline (Amitrip) (200 μ M) or corticosterone (Cortico) (100 μ M). Data
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20 are expressed as % of substrate accumulation found in control cells, arbitrarily set at 100%, and
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22 are the means \pm SD of three independent assays, each being performed in triplicate. ***, $p <$
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24 0.001, when compared to control cells. (B) Effects of various concentrations of ruxolitinib on
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26 OCT2 activity were analyzed in HEK-OCT2 cells with or without pre-incubation with
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28 ruxolitinib. Data are expressed as % of OCT2 activity in control cells not exposed to ruxolitinib,
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30 arbitrarily set at 100%; they are the means \pm SD of three independent assays. IC₅₀ values are
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32 indicated at the top of the graph. (C) Accumulation of ruxolitinib in HEK-OCT2 and HEK-
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34 MOCK cells exposed or not to 200 μ M amitriptyline was determined by LC-MS/MS. Data are
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36 the means \pm SD of three independent assays, each being performed in triplicate. NS, not
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38 statistically significant.
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47 **Figure 4.** Interactions of ruxolitinib with MATEs.

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49 (A) Accumulation of the reference MATE substrate TEA was determined in HEK-MATE cells
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51 incubated in the absence (control) or presence of ruxolitinib (10 or 100 μ M) or of the reference
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53 MATE inhibitor verapamil (Vera) (200 μ M). Data are expressed as % of substrate accumulation
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55 found in control cells, arbitrarily set at 100%, and are the means \pm SD of three independent
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57 assays, each being performed in triplicate. ***, $p <$ 0.001, when compared to control cells. (B)
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3 Effects of various concentrations of ruxolitinib on MATE1 and MATE2-K activity were
4 analyzed in HEK-MATE cells with or without pre-incubation with ruxolitinib. Data are
5 expressed as % of MATE activity in control cells not exposed to ruxolitinib, arbitrarily set at
6 100%; they are the means \pm SD of three independent assays. IC₅₀ values are indicated at the top
7 of the graphs. (C) Accumulation of ruxolitinib in HEK-MATE1, HEK-MATE2-K and HEK-
8 MOCK cells exposed or not to 200 μ M verapamil was determined by LC-MS/MS. Data are the
9 means \pm SD of three independent assays, each being performed in triplicate. *, p < 0.05, **, p
10 < 0.01 and NS, not statistically significant.
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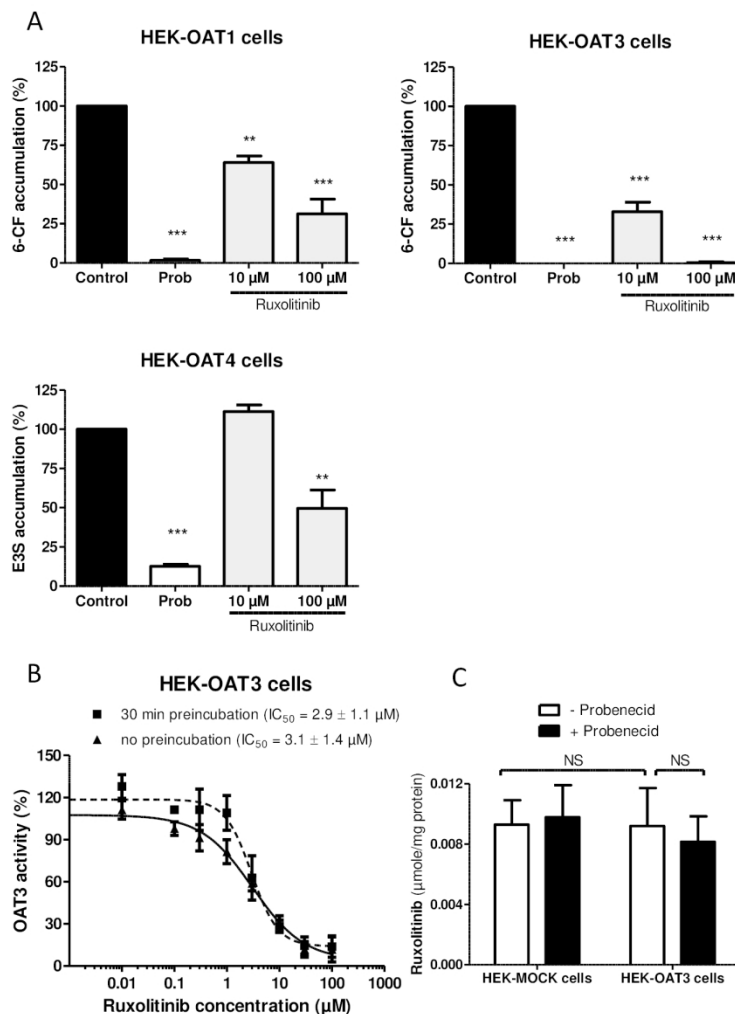
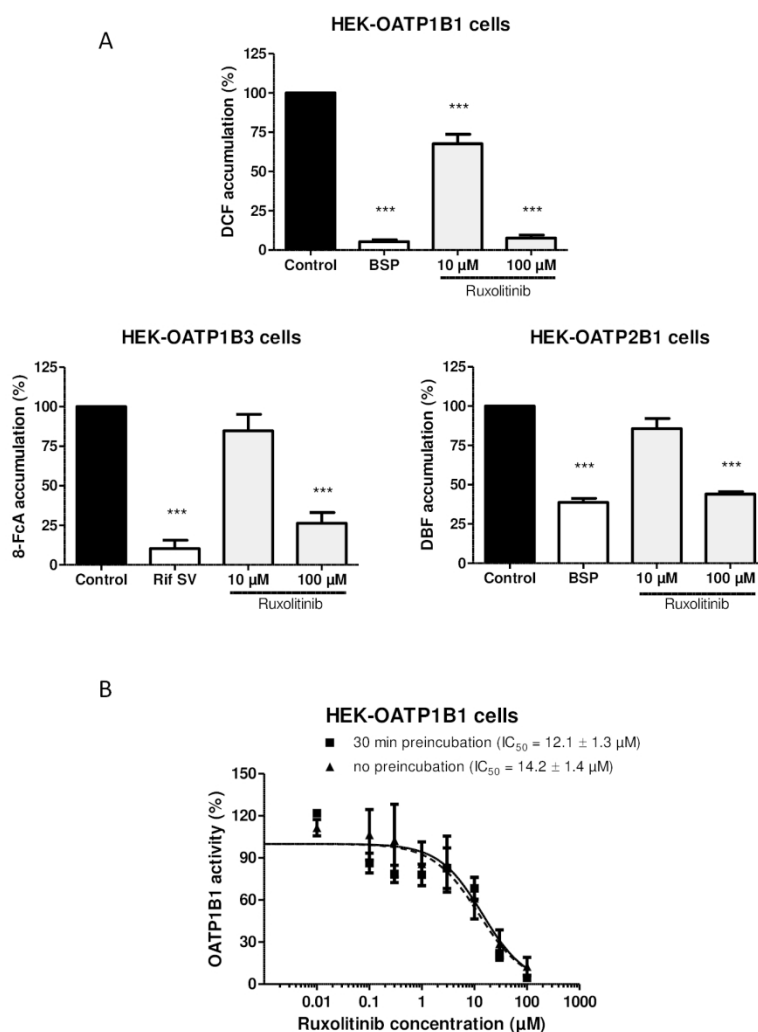


Figure 1. Interactions of ruxolitinib with OATs.

(A) Accumulation of reference substrates (6-CF for OAT1 and OAT3, E3S for OAT4) was determined in HEK-OAT cells incubated in the absence (control) or presence of ruxolitinib (10 or 100 μ M) or of the reference OAT inhibitor probenecid (Prob) (2 mM). Data are expressed as % of substrate accumulation found in control cells, arbitrarily set at 100%, and are the means \pm SD of three independent assays, each being performed in triplicate. **, $p < 0.01$ and ***, $p < 0.001$, when compared to control cells. (B) Effects of various concentrations of ruxolitinib on 6-CF-related OAT3 activity were analyzed in HEK-OAT3 cells with or without pre-incubation. Data are expressed as % of OAT3 activity in control cells not exposed to ruxolitinib, arbitrarily set at 100%; they are the means \pm SD of three independent assays. IC₅₀ values are indicated at the top of the graph (C) Accumulation of ruxolitinib in HEK-OAT3 and HEK-MOCK cells exposed or not to 2 mM probenecid was determined by LC-MS/MS. Data are the means \pm SD of three independent assays, each being performed in triplicate. NS, not statistically significant.

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45 Figure 2. Effects of ruxolitinib on OATP activities.

46 (A) Accumulation of reference substrates (DCF for OATP1B1, 8-FcA for OATP1B3, DBF for OATP2B1) was
 47 determined in HEK-OATP cells incubated in the absence (control) or presence of ruxolitinib (10 or 100 μM)
 48 or of the reference OATP inhibitors BSP (100 μM) or rifamycin SV (Rif SV) (100 μM). Data are expressed as
 49 % of substrate accumulation found in control cells, arbitrarily set at 100%, and are the means \pm SD of three
 50 independent assays, each being performed in triplicate. *, $p < 0.05$ and ***, $p < 0.001$, when compared to
 51 control cells. (B) Effects of various concentrations of ruxolitinib on DCF-related OATP1B1 activity were
 52 analyzed in HEK-OATP1B1 cells with or without pre-incubation with ruxolitinib. Data are expressed as % of
 53 OATP1B1 activity in control cells not exposed to ruxolitinib, arbitrarily set at 100%; they are the means \pm
 54 SD of three independent assays. IC_{50} values are indicated at the top of the graph.

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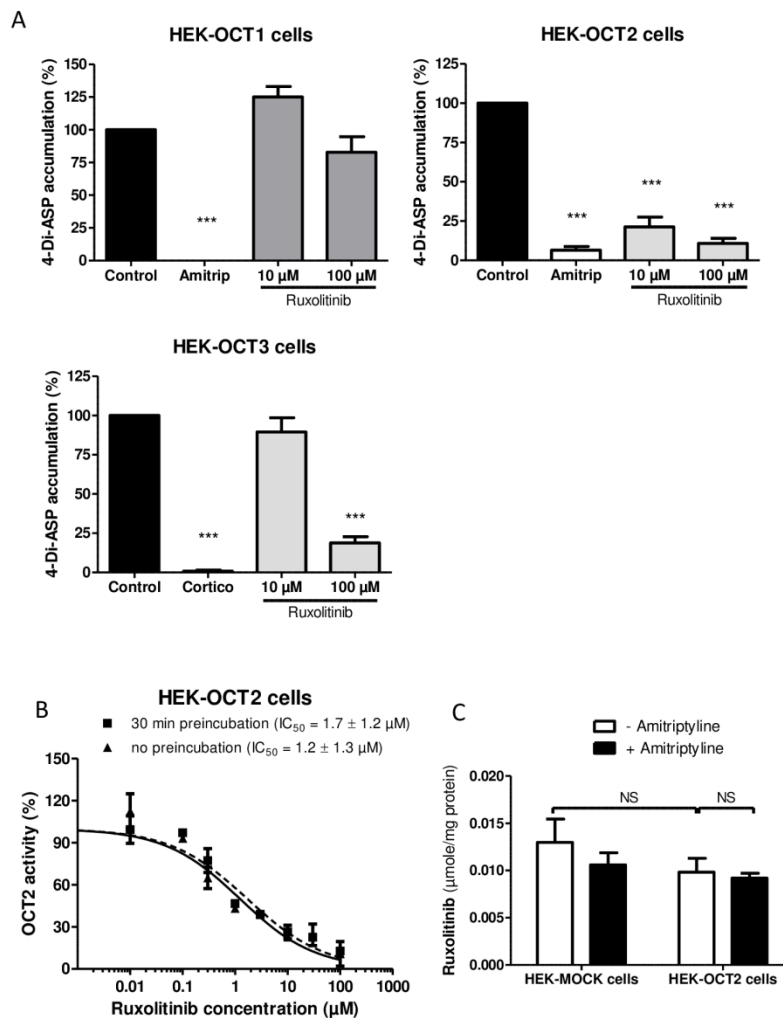


Figure 3. Interactions of ruxolitinib with OCTs.

(A) Accumulation of the reference OCT substrate 4-Di-ASP was determined in HEK-OCT cells incubated in the absence (control) or presence of ruxolitinib (10 or 100 μ M) or of the reference OCT inhibitors amitriptyline (Amitrip) (200 μ M) or corticosterone (Cortico) (100 μ M). Data are expressed as % of substrate accumulation found in control cells, arbitrarily set at 100%, and are the means \pm SD of three independent assays, each being performed in triplicate. ***, $p < 0.001$, when compared to control cells. (B) Effects of various concentrations of ruxolitinib on OCT2 activity were analyzed in HEK-OCT2 cells with or without preincubation with ruxolitinib. Data are expressed as % of OCT2 activity in control cells not exposed to ruxolitinib, arbitrarily set at 100%; they are the means \pm SD of three independent assays. IC₅₀ values are indicated at the top of the graph. (C) Accumulation of ruxolitinib in HEK-OCT2 and HEK-MOCK cells exposed or not to 200 μ M amitriptyline was determined by LC-MS/MS. Data are the means \pm SD of three independent assays, each being performed in triplicate. NS, not statistically significant.

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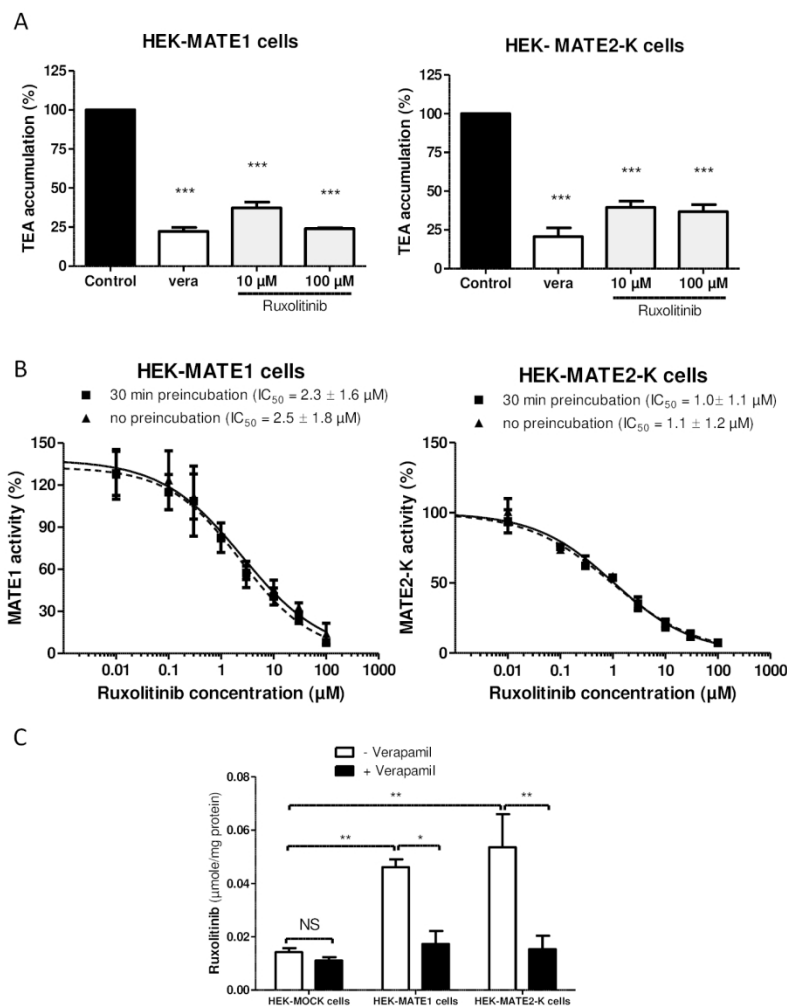


Figure 4. Interactions of ruxolitinib with MATEs.

(A) Accumulation of the reference MATE substrate TEA was determined in HEK-MATE cells incubated in the absence (control) or presence of ruxolitinib (10 or 100 μM) or of the reference MATE inhibitor verapamil (Vera) (200 μM). Data are expressed as % of substrate accumulation found in control cells, arbitrarily set at 100%, and are the means \pm SD of three independent assays, each being performed in triplicate. ***, $p < 0.001$, when compared to control cells. (B) Effects of various concentrations of ruxolitinib on MATE1 and MATE2-K activity were analyzed in HEK-MATE cells with or without pre-incubation with ruxolitinib. Data are expressed as % of MATE activity in control cells not exposed to ruxolitinib, arbitrarily set at 100%; they are the means \pm SD of three independent assays. IC_{50} values are indicated at the top of the graphs. (C) Accumulation of ruxolitinib in HEK-MATE1, HEK-MATE2-K and HEK-MOCK cells exposed or not to 200 μM verapamil was determined by LC-MS/MS. Data are the means \pm SD of three independent assays, each being performed in triplicate. *, $p < 0.05$, **, $p < 0.01$ and NS, not statistically significant.

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Table 1: Summary of experimental conditions used for transporter activity assays

Transporter	Gene symbol	Cells	Substrate Method of analysis	Reference inhibitor
OATP1B1	<i>SLCO1B1</i>	HEK-OATP1B1	DCF (10.0 μ M) Fluorimetry (492/517 nm)*	BSP (100 μ M)
OATP1B3	<i>SLCO1B3</i>	HEK-OATP1B3	8-FcA (10.0 μ M) Fluorimetry (485/535 nm)	BSP (100 μ M)
OATP2B1	<i>SLCO2B1</i>	HEK-OATP2B1	DBF (10.0 μ M) Fluorimetry (485/535 nm)	Rifamycin SV (100 μ M)
OAT1	<i>SLC22A6</i>	HEK-OAT1	6-CF (10.0 μ M) Fluorimetry (492/517 nm)	Probenecid (2 mM)
OAT3	<i>SLC22A8</i>	HEK-OAT3	6-CF (10.0 μ M) Fluorimetry (492/517 nm)	Probenecid (2 mM)
OAT4	<i>SLC22A11</i>	HEK-OAT4	[³ H]-E3S (4.4 nM) Scintillation counting	Probenecid (2 mM)
OCT1	<i>SLC22A1</i>	HEK-OCT1	4-Di-ASP (10.0 μ M) Fluorimetry (485/607 nm)	Amitriptyline (200 μ M)
OCT2	<i>SLC22A2</i>	HEK-OCT2	4-Di-ASP (10.0 μ M) Fluorimetry (485/607 nm)	Amitriptyline (200 μ M)
MATE1	<i>SLC47A1</i>	HEK-MATE1	TEA (40.0 μ M) LC-MS/MS	Verapamil (200 μ M)
MATE2-K	<i>SLC47A2</i>	HEK-MATE2	TEA (40.0 μ M) LC-MS/MS	Verapamil (200 μ M)

Abbreviations: 6-CF, 6-carboxyfluorescein; 4-Di-ASP, 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide; BSP, sulfobromophthalein; DBF, 4',5'-dibromofluorescein; DCF, 2',7'-dichlorofluorescein; E3S, [6,7-3H(N)]-estrone-3-sulfate; LC-MS/MS, liquid chromatography-tandem mass spectroscopy; MATE, multidrug and toxin extrusion; OAT, organic anion transporter; OATP, organic anion transporter polypeptide; OCT, organic cation transporter; TEA, tetraethylammonium.

* Correspond to excitation/emission wavelengths.

Table 2: Prediction for *in vivo* inhibition of SLC transporter activity by ruxolitinib

Transporter	Ruxolitinib		IC ₅₀ ^c	I _{max,u} /IC ₅₀	R value	Potential <i>in vivo</i> inhibition ^d
	I _{max,u} (μM) ^a	I _{in,max} (μM) ^b				
OAT1	0.216		> 10.0 μM	< 0.02		No inhibition
OAT3			3.1 μM	0.07		No inhibition
OAT4			> 10.0 μM	< 0.02		No inhibition
OATP1B1	17.7		> 10.0 μM		1.05	No inhibition
OATP1B3			> 10.0 μM		1.05	No inhibition
OATP2B1			> 10.0 μM		1.05	No inhibition
OCT1			> 100.0 μM		1.01	No inhibition
OCT2	0.216		1.4 μM	0.15		Risk of inhibition
OCT3			> 10.0 μM	< 0.02		No inhibition
MATE1			2.5 μM	0.09		No inhibition
MATE2K			1.1 μM	0.20		Risk of inhibition

^aI_{max,u} was calculated based on *in vivo* I_{max} (7.2 μM) and unbound fraction (0.03) described in US FDA data (Center for Drug Evaluation and Research 2011).

^bI_{in,max} was calculated with I_{max} value of 7.2 μM for a dosing of 200 mg according to Materials and Methods equation F and data from Umehara *et al.* 2019 (F_a = 0.99, F_g = 0.87, k_a = 2.02 h⁻¹, R_B = 1.2, Q_h = 90 L/h).

^cIC₅₀ values from the present study .

^ddefined and adapted according to the US FDA criteria for transporter-related DDI (based on equation D and E of Materials and Methods section). For US FDA-non regulatory transporters (OCT1, OAT4, OATP2B1 and OCT3), the equation D was applied for OAT4 and OCT3 and the equation E for OATP2B1 and OCT1.

Table 3. Comparison of data related to ruxolitinib-mediated *in vitro* inhibition of SLC transporters from the present study and from the FDA report for ruxolitinib (Center for Drug Evaluation and Research 2011).

Transporter	FDA report		Present study	
	Probe substrate ^a	Ruxolitinib effect	Probe substrate ^a	Ruxolitinib effect
OAT1	Aminohippuric acid	No inhibition (up to 37.5 μ M)	6-CF	Inhibition ($IC_{50}>10.0$ μ M)
OAT3	Estrone-3 sulfate	Inhibition ($IC_{50}=6.5$ μ M)	6-CF	Inhibition ($IC_{50}=3.1$ μ M)
OATP1B1	Estradiol-17 β glucuronide	Inhibition ($IC_{50}=19.3$ μ M)	DCF	Inhibition ($IC_{50}>10.0$ μ M)
OATP1B3	Estradiol-17 β glucuronide	Inhibition ($IC_{50}=20.5$ μ M)	8-FcA	Inhibition ($IC_{50}>10.0$ μ M)
OCT1	1-methyl-4-phenylpyridinium	Inhibition ($IC_{50}=9.1$ μ M)	4-Di-ASP	No inhibition (up to 100.0 μ M)
OCT2	1-methyl-4-phenylpyridinium	Inhibition ($IC_{50}=9.8$ μ M)	4-Di-ASP	Inhibition ($IC_{50}=1.2$ μ M)

^aFor each condition of IC_{50} determination, the used probe substrate is specified.

Supplemental data

Xenobiotica

Differential in vitro interactions of the Janus kinase inhibitor ruxolitinib with human SLC drug transporters

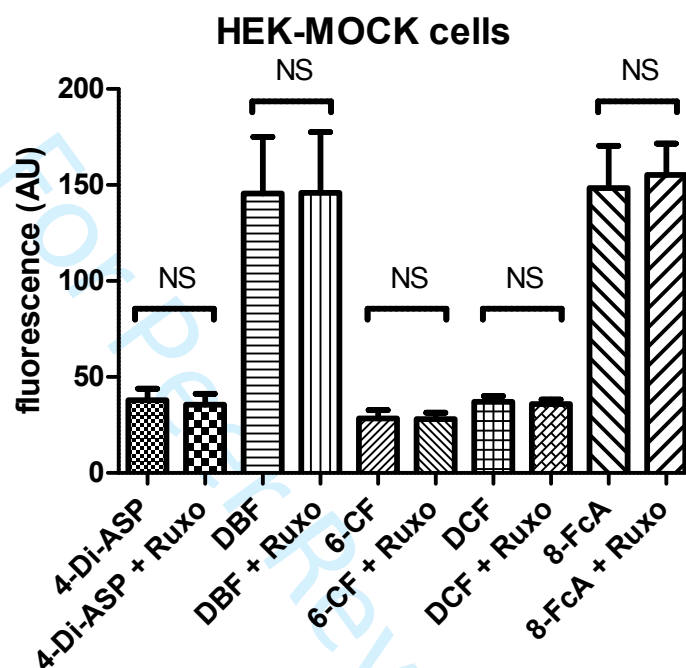


Figure S1: Lack of major analytical interaction of ruxolitinib (Ruxo) with fluorescent probes used as substrates for transporters. HEK-MOCK cells plated in 96 wells were either pre-treated or not with 100 μ M ruxolitinib for 3 h; they were next incubated with the reference fluorescent probes 4 Di-ASP, DCF, DBF, 6-CF or 8-FcA (each at 10 μ M) for 5 min. Probe-related fluorescence was then determined by spectrofluorimetry; data are expressed as arbitrary unit (AU)/wells and are the mean \pm SEM of at least three assays. NS, not statistically significant.