Interleukin-2 treatment effect on imatinib pharmacokinetic, P-gp and BCRP expression in mice

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The aim of this study was to investigate the effect that recombinant interleukin-2 (rIL-2) (0.16 MUI/injection) had on the pharmacokinetics of imatinib (IM) in plasma. In this study, IM was given orally to mice at a dose of 150 mg/kg once a day for 11 days (from day 1 to 11) either alone or in combination with intraperitoneal injections of rIL-2 twice a day from day 8 to 11. Pharmacokinetic parameters were determined using WinNonLin software. Areas under the curve were compared using Bailer's method. The repeated administration of the rIL-2 + IM combination was shown to have two pharmacokinetic advantages compared with repeated IM doses alone. In addition to the pharmacodynamic interest of this treatment, we found that the combined treatment significantly increased the IM C_{max} ($P<0.05$) and significantly increased the IM trough concentration ($C_{24\,h}$) ($P<0.01$), which was always above the minimum therapeutic IM concentration (1 μmol/l) in plasma. Those pharmacokinetic modifications may be explained, in part, by a decrease in the P-glycoprotein expression in the three intestinal segments of the mice (duodenum, $P<0.01$; jejunum, $P<0.05$; and ileum, $P<0.05$) and a decrease in BCRP expression in the duodenum segment ($P<0.05$) due to rIL-2. In another experiment, we found a significant induction of intestinal P-glycoprotein expression in mice that had been given IM orally (150 mg/kg) twice a day for 11 days. It would be interesting to further investigate the IM disposition associated with rIL-2 treatment for clinical applications. *Anti-Cancer Drugs* **21:193–201 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Recently, a study reported on an established B16F10 lung metastases model in C57Bl/6 mice that the imatinib (IM) + recombinant interleukin-2 (rIL-2) treatment significantly decreased the number of lung metastases as compared with the IM alone group [1]. Another study reported increased anticancer activity of a newly identified dendritic cell subset involved in tumour immunosurveillance [interferon (IFN)-producing killer dendritic cells or (IKDC)] in mice after rIL-2 + IM administration. This study showed the synergy between IM and rIL-2 in increasing IKDC anticancer activity in several tumour models. rIL-2 + IM induced the activation and proliferation of IKDC and led to their accumulation at in-vivo tumour sites in mice [2]. The mechanism of the synergy between IL-2 and IM is not fully known. Our hypothesis is that the BCR-ABL oncogene is involved in several signal transduction pathways: (i) BCR-ABL interacts with the growth factor receptor-bound protein-2, which activates the protein RAS/MAPK; (ii) BCR-ABL also interacts with the phosphatidyl inositol 3-kinase and inhibits apoptosis; and (iii) BCR-ABL interacts with the JAK/STAT pathway, which induces cell proliferation [3,4]. BCR-ABL binds to and interacts with all these proteins by inducing a macromolecular complex. IM is active in chronic myeloid leukaemia (CML) in binding BCR-ABL. The signalling pathways induced by BCR-ABL and IL-2 are similar [4,5]. When IL-2 binds to its receptor, the RAF/MAPK and JAK/STAT pathways are induced, and as a result, competition between BCR-ABL and IL-2-induced pathways could arise, which could explain the synergy of the IM + IL-2 combination.

IM (Glivec; Novartis, Rueil-Malmaison, France) is a selective tyrosine kinase inhibitor that has been successfully used in treating CML and gastrointestinal stromal tumours (GISTs). The deregulation of the tyrosine kinase activity of the BCR-ABL fusion protein has been linked to the oncogenesis of CML and acute lymphoid leukaemia [6]. The tyrosine kinase inhibitor has also been used in combination with IFN-α in patients with acute lymphoblastic leukaemia that have developed resistance to IM or dasatinib monotherapy [7]. Preclinical studies have provided evidence for the efficacy of IM in treating mice bearing non-small cell lung carcinoma xenografts [8,9]. IM is transported by human and rodent ATP-binding cassette transporters such as P-glycoprotein (P-gp) (ABCB1) and BCRP (ABCG2) [10]. Recent studies have provided evidence that IM transport at the

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mouse blood-brain barrier is limited by ABCB1 and ABCG2 [11,12]. ABCB1 and ABCG2, which are well-known modulators of anticancer agent pharmacokinetics, lead to an increase of drug efflux and a reduction of drug accumulation in tumour cells. Enhanced expression of ABCB1 is considered to be a major mechanism of paclitaxel resistance [13]. In addition, intestinal ABCB1 reduces oral bioavailability of drugs that are substrates of the pump such as vincristine and paclitaxel [14]. Glivec is orally administered because of its high oral bioavailability (98%) [15]. Preliminary data, however, revealed a reduced systemic exposure to IM in GIST patients when given for prolonged periods of time [16]. Furthermore, the systemic exposure of IM shows wide interindividual variability in chronic myeloid leukaemia and GIST patients [16,17]. Oral bioavailability is highly dependent on gastrointestinal absorption and first-pass drug metabolism, two processes that vary considerably among individuals [18]. In particular, the intended chronic use of oral IM may induce enhanced expression of intestinal drug transport pumps (ABCB1 and ABCG2) and drug-metabolizing enzymes, which may then limit the bioavailability and efficacy of IM. This would be in agreement with the enhanced ABCB1 and ABCG2 expression observed in Caco-cells after IM exposure [19]. Moreover, this may eventually lead to decreased drug uptake and lower drug plasma levels, the development of cellular resistance and subsequent treatment failure. This description of pharmacokinetic resistance may have important consequences in the treatment approach for IM. Despite the excellent efficacy of IM in CML, the response in patients is heterogeneous, which may in part be caused by pharmacogenetic variability. Dulucq *et al.* [20] analysed the three most relevant single nucleotide polymorphisms of the *ABCB1* gene in patients with CML (1236C > T; 2677G > T/A and 3435C > T) and found that among the patients who are homozygous for the 1236T allele, 85% achieved a major molecular response versus 47.7% for the other genotypes. Another study also reported that trough IM plasma concentration was associated with both cytogenetic and molecular responses to standard-dose IM in CML [21].

rIL-2 is used to treat patients with renal adenocarcinoma with metastasis. A study provided evidence for the efficacy of inhaled rIL-2 therapy to treat lung metastasis in patients with malignant melanoma [22]. In this study, 13 patients of 27 treated with inhaled rIL-2, experienced a complete or partial pulmonary remission without significant side effects. In addition, it has been shown that rIL-2 decreases MDR1 mRNA as well as P-gp expression in cultured cells from human colon carcinoma [23].

Furthermore, we have shown earlier that rIL-2 at a dose of 0.27 MUI (given intraperitoneally) effectively decreased P-gp activity in the intestine of Swiss mice [24]. As it has been shown that the efficacy of tumour treatment is increased by combining conventional chemotherapeutic drugs with cytokines in experimental and human malignancy [25], it appeared of great interest to investigate the interaction between P-gp and cytokines. Several cytokines such as proinflammatory cytokines and rIL-2 have been found to modulate P-gp expression in several experimental models. The influence of 100 UI/ml of cytokines (interferon-γ, TNF-α or rIL-2) on the *MDR1* gene and P-gp expression was investigated in the human colon carcinoma cell lines HCT115 and HCT16 [23]. In both cell lines, MDR1 mRNA levels were decreased by all three cytokines in a time-dependent manner. At 72 h after cytokine treatment, MDR1 levels were back to control levels. The expression of P-gp was also decreased, which was in agreement with the reduction observed in the transcripts.

We combined IM and IL-2 for several reasons. First, IL-2 is instrumental in creating an immune response. It stimulates NK cells and cytotoxic T lymphocyte activity. Second, IM is an ABCB1 and ABCG2 substrate known to be involved in IM resistance; however, this is not the only mechanism of resistance. On the basis of the premise that the IM pharmacokinetic parameters could be modified as a result of diminished ABCB1 and ABCG2 expression through rIL-2 treatment, we evaluated the pharmacokinetics of combining rIL-2 immunotherapy and a tyrosine kinase inhibitor.

The aim of this study was to document the effect of IM treatment (orally administered) on the intestinal ABCB1 and ABCG2 expression in mice by western blot analysis. We also investigated whether rIL-2 pretreatment modified the IM disposition in plasma in mice after IM oral administration once a day for 11 days. Furthermore, we also examined the influence that rIL-2 had on the intestinal ABCB1 and ABCG2 expression in mice by western blot analysis.

Materials and methods

Chemicals

Cell culture media and reagents were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, California, USA). IM (Glivec) stock solution (5 mg/ml) was obtained by dissolving IM powder (Sigma-Aldrich, Saint-Quentin Fallavier, France) in methanol. Throughout the study, water was used for injections (C.D.M. Lavoisier, Paris, France).

Acetonitrile, tert-butyl methyl ether, trifluoroacetic acid, tetrabutyl ammonium and methanol (high-performance liquid chromatography grade) were purchased from VWR (Fontenay sous Bois, France).

Drugs and treatment

Proleukin (rIL-2) was purchased from Chiron (Suresnes, France). One millilitre of the reconstituted rIL-2 solution was diluted with a 5% dextrose solution to obtain a final concentration of 1.8 MUI/ml.

IM (Glivec) solution (20 mg/ml) was obtained by dissolving a tablet of 100 mg (Novartis) in water, and then the solution was given orally (150 mg/kg).

Animals

Animal experiments were carried out in compliance with French and European regulations. Isoflurane anaesthesia was induced at 5% and maintained at 2.5% in air. Female C57Bl/6 mice (Charles River, France), 6–8 weeks old and weighing 20–25 g, were used. They were given water and food *ad libitum* in accordance to the European Community guidelines. The animals were housed under these conditions for at least 1 week before being used.

Imatinib pharmacokinetic studies in mice after oral administration

Treatment, sampling time and collection

Three groups of mice were formed. In the first group, mice received a single dose of IM (150 mg/kg) orally (group 1). In the second group, mice received 150 mg/kg of IM orally once a day from day 1 to 11 (group 2). In the third group, from day 8 to 11, mice received 0.16 MUI (or 10 µg) of rIL-2 by intraperitoneal injections twice daily, and from day 1 to 11, 150 mg/kg once a day of IM orally (group 3). Throughout the manuscript, the treatment groups will be referred to as group 1, group 2 and group 3. For the IM assay, blood samples were collected in heparinized tubes before IM administration and at 0.5, 1, 2, 3, 6, 8, 16, 24, 30 and 48 h after a single IM administration, using four mice per time point. For the two other IM assays (once a day for 11 days either alone or with rIL-2 treatment), blood samples were collected in heparinized tubes before IM administration and at 0.5, 1, 2, 3, 6, 8, 16 and 24 h after the last IM administration, using four mice per time point. Blood samples were centrifuged for 10 min at 3500 rpm, and the plasma was harvested into clean glass tubes and stored at −20°C until analysis.

Imatinib quantification in plasma

IM plasma concentrations were measured using a validated 'reverse-phase high-performance liquid chromatography/UV' method. This quantification followed a liquid-phase extraction. For analysis, 100 µl of the plasma sample was mixed with 200 µl of acetonitrile. The mixture was vortexed and centrifuged (10 000 rpm/min). The supernatant was evaporated under nitrogen at 40°C. The residue was reconstituted in 600 µl of sodium carbonate (Na_2CO_3 0.2 M), vortexed and mixed with 3 ml of tert-butyl methyl ether. The mixture was agitated for 10 min, and then the tert-butyl methyl ether was evaporated under nitrogen at 40°C. Finally, the residue was reconstituted in 200 µl of mobile phase (tetrabutyl 75%, acetonitrile 15% and methanol 10%) and vortexed for 20 s. An aliquot of 50 µl was then injected into the chromatographic system.

Chromatographic analysis was accomplished using a Nucleosil C_{18} column (4.6 × 125 mm, 3 µ) (Interchim, Montlucon, France) with a mobile phase delivered at a flow rate of 1.2 ml/min. The eluent was monitored at 260 nm. The quantification method was validated according to Food and Drug Administration recommendations [26].

The IM standard curve was well described by $1/X^2$ concentration weighted least-square linear regression. Over an IM plasma concentration range of 100–10 000 ng/ml, the determination coefficient R^2 of the calibration curves remained greater than 0.99. On the basis of quality control samples, the overall relative standard deviation (an index of precision) was less than 12%. The overall relative error (an index of accuracy) was less than 10%. The lower limit of quantification was 100 ng/ml. The stability of plasma samples at −20°C was confirmed by analysing six quality control samples at the end of the study.

Calibration standards of IM were prepared in drug-free human plasma by spiking with concentrated standards to obtain a concentration range between 100 and 10 000 ng/ml. Quality control samples for IM were prepared in drug-free human plasma by spiking with concentrated standards. Three levels of quality control were prepared, a low level at 300 ng/ml, a medium level at 1500 ng/ml and a high level at 7500 ng/ml. IM was extracted from 100 µl of plasma for quality control or calibration standards.

Data analysis

As each animal provided only one sample of blood, data from animals of the same group were pooled using a naive averaging data approach [27]. Data were analysed separately for each treatment using the average concentration at each time point. The noncompartmental analysis was performed using WinNonlin professional Version 4.1 software (Pharsight Mountain View, California, USA).

The mean maximum concentration (C_{max}), the time necessary to reach it (T_{max}) and the mean minimum concentration (trough concentration) were evaluated from experimental curves. IM terminal half-lives ($t_{1/2}$) were calculated from the elimination rate constant (K_e). K_e was estimated as the slope of the log-linear terminal portion of the plasma concentration versus the time curve, determined using unweighted linear least square regression analysis. The best number of concentration was chosen as that giving the highest coefficient of determination as recommended. The number of time concentration point considered to calculate the elimination half-life was chosen by Winonlin software to obtain the highest coefficient of determination as recommended.

The mean area under the concentration-time curves ($AUCs_{0\rightarrow\alpha}$) were calculated by the trapezoidal method from zero to the last concentration time point (T_{last}) and extrapolated from T_{last} to infinity using the equation: $AUCs_{0\rightarrow\alpha}=AUCs_{0\rightarrow last}+C_{last}/K_e$, in which C_{last} is the mean of the last quantified concentration above the lower limit of quantification.

Semiquantitative determination of intestinal P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) expression by western blot analysis

Intestine samples were homogenized using a glass teflon potter in a buffer (triethanolamine 10 mmol/l + 8.5% of saccharose) containing protease inhibitors. The crude membranes obtained were solubilized with lysis buffer (Tris 1 mol/l, EDTA 0.5 mol/l, NaCl 3 mol/l, Triton 10%, SDS 20% and protease inhibitor). Protein concentration was determined using the colometric Bicinchonimic Assay kit (Sigma-Aldrich, Saint-Quentin Fallavier, France) with bovine serum albumin as a standard. Fifteen micrograms of proteins were separated by SDS-polyacrylamide gel electrophoresis on an 8% polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham, Orsay, France). The nitrocellulose membranes were then incubated with a primary antibody (C219, diluted to 1 : 100, DAKO, Denmark), washed and finally incubated with a peroxidase-conjugated anti-mouse IgG secondary antibody. The nitrocellulose membranes were also incubated with the primary antibody (BXP-53, diluted 1 : 40, Alexis Biomedical, Pennsylvania, USA). The immunoreactive bands were visualized by the Enhanced Chimio-Luminescent system (Perkin ElmerLife Science, Boston, Massachusetts, USA). The nitrocellulose membranes were also incubated with anti-β-actin (diluted to 1: 5000, Sigma, St Louis, USA) to normalize ABCB1 and ABCG2 expression. The autoradiographs of ABCB1, ABCG2 and β-actin protein were scanned and analysed by densitometry using the Scion Image program to obtain a quantitative evaluation of the levels in the three intestine segments.

In the pharmacokinetic study (Figs 2 and 3)

ABCB1 and ABCG2 expression was measured in three intestine segments (duodenum, jejunum and ileum) by western blot analysis. The intestine was removed (and stored at −80°C until analysis) 1 h after the last IM administration from six female C57Bl/6 mice that had been given rIL-2 at a dose of 10 μg twice a day for three days and IM (150 mg/kg) once a day for 11 days and from six female C57Bl/6 mice that had been given IM alone (150 mg/kg once a day for 11 days).

Imatinib effect on ABCB1 and ABCG2 expression (Fig. 4)

ABCB1 and ABCG2 expression was measured in the duodenum part of the intestine only by western blot analysis. The intestine was removed (and stored at −80°C until analysis) 1 h after the last IM administration from four female C57Bl/6 mice, which had been given IM (150 mg/kg) twice a day for 11 days and from 4 control mice (without treatment).

Statistical analysis

Pharmacokinetic study

C_{max} and trough concentration were compared using Student's *t*-test. $AUCs_{0\rightarrow T_{last}}$ of the three groups (rIL-2 + IM repeated doses, IM alone repeated doses and IM unique dose) were compared using Bailer's method [28].

Western blot study

In the western blot analysis of the ABCB1 and ABCG2 expression, the mean ABCB1/β-actin ratio and ABCG2/β-actin ratio ± SEM (n=4 to 6) were compared with the Mann–Whitney test.

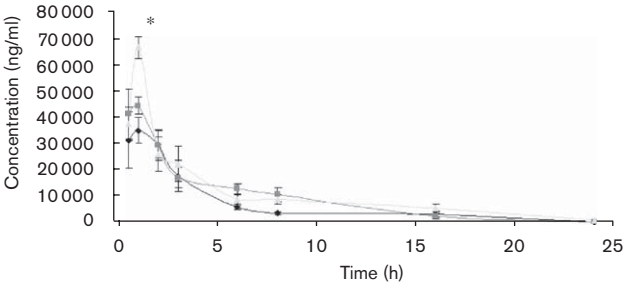
Results

Noncompartmental analysis in plasma after imatinib oral administration

The plasma IM concentration versus time profile after oral administration is shown in Fig. 1. The main pharmacokinetic parameters in plasma after oral administration obtained after a noncompartmental analysis are summarized in Table 1.

The plasma IM maximal concentration (C_{max}) in the rIL-2 + IM (repeated doses) group was significantly (1.5-fold) higher than in the IM alone (repeated doses) group ($P < 0.05$; Student's *t*-test). Furthermore, the trough concentration (plasma concentration at the end of the dose interval of 24 h) was significantly higher in the rIL-2 + IM (repeated doses) group than in the IM alone (repeated doses) group ($P < 0.01$; Student's *t*-test).

Fig. 1



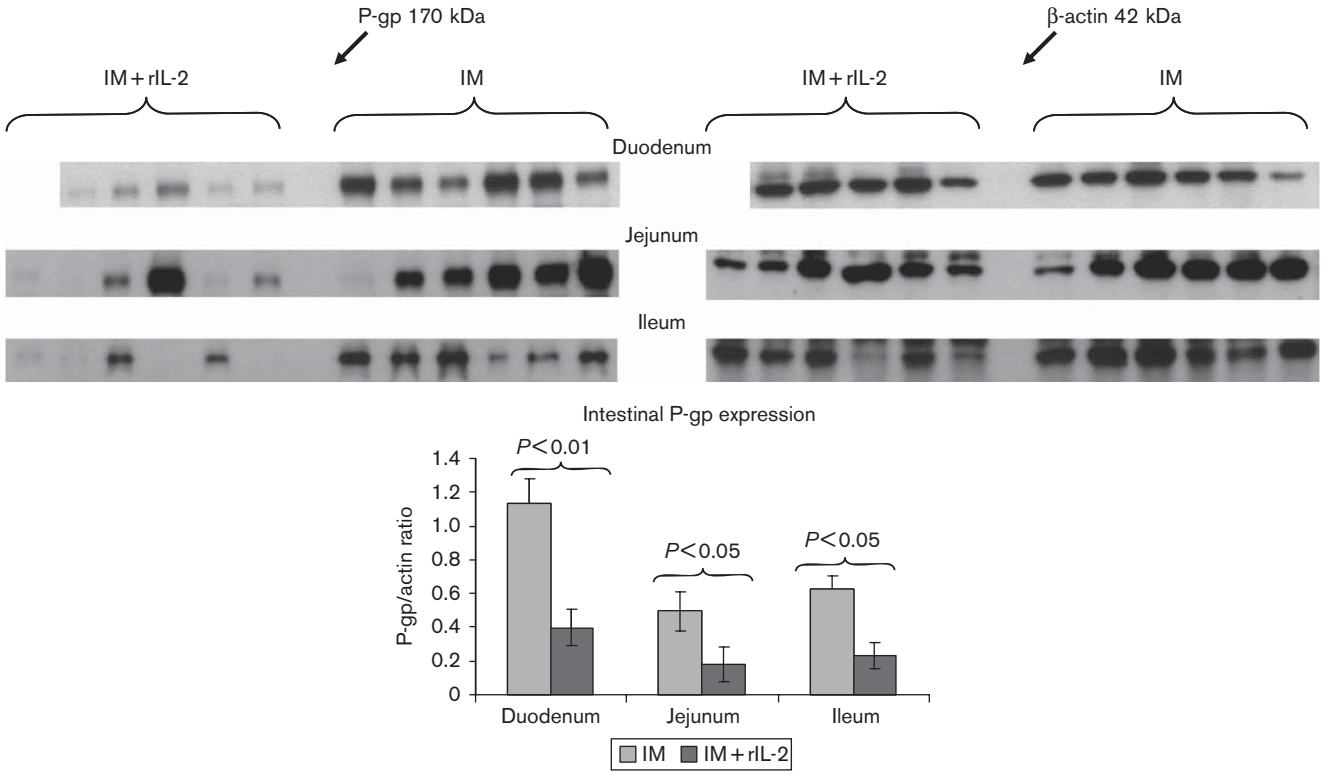
Mean imatinib (IM) concentration versus time profiles in plasma: IM alone per os at 150 mg/kg/day unique dose (diamonds = group 1); IM alone per os at 150 mg/kg/day repeated dose (for 11 days) (squares = group 2); IM per os at 150 mg/kg/day repeated dose (for 11 days) + 3 days treatment with recombinant interleukin-2 at 0.16 MUI twice a day from day 8 to 11 (triangles = group 3). Data represent the mean IM concentration ± mean standard error in plasma of four mice per time point. The concentration time profile shown is a 0–24-h interval on day 1 for IM alone (group 1) and 0–24-h interval on day 11 for the other 2 groups. *Significantly higher IM C_{max} in group 3 as compared with IM C_{max} in group 2.

Table 1 Imatinib pharmacokinetic parameters after oral administration with noncompartmental analysis

	IM single dose	IM repeated doses	rIL-2 + IM repeated doses
T_{max} (h)	1	1	1
C_{max} (ng/ml) × 10 000	0.35 ± 0.05	0.44 ± 0.03[a]	0.67 ± 0.04[a]
$AUC_{0\to 24\,h}$ (ng h/ml) × 10 000		2.21 ± 0.20[b]	2.50 ± 0.23[c]
$AUC_{0\to\infty}$ (ng h/ml) × 10 000	1.63 ± 0.18[b,c]		
$T_{1/2}$ (h)	3.35	3.05	4.57
Trough concentration (ng/ml at time 24 h or $C_{24\,h}$)	150 ± 68	146 ± 57[d]	615 ± 37[d]

IM AUC between the three groups was compared with Bailer's method in the plasma compartment.
AUC, area under the curve; IM, imatinib; rIL-2, recombinant interleukin-2.
[a]C_{max} comparison between the groups IM alone repeated doses vs. rIL2 + IM in the plasma compartment; $P<0.05$ (Student's *t*-test).
[b]$AUC_{0\to\infty}/AUC_{0\to 24\,h}$ comparison between the groups IM alone single dose vs. IM alone repeated doses in the plasma compartment; $P<0.05$ (Bailer's method).
[c]$AUC_{0\to\infty}/AUC_{0\to 24\,h}$ comparison between the groups IM alone single dose vs. rIL2 + IM in the plasma compartment; $P<0.01$ (Bailer's method).
[d]$C_{24\,h}$ comparison between the groups IM alone repeated doses vs. rIL2 + IM in the plasma compartment; $P<0.01$ (Student's *t*-test).

Fig. 2



ABCB1 immunodetection from the intestine segments of mice treated by recombinant interleukin-2 (rIL-2) + imatinib (IM) or IM alone. The mean ABCB1/actin ratio ± mean standard error from six intestines of mice per group are represented in the histogram. P-gp, P-glycoprotein.

The plasma IM total exposure measured by $AUCs_{0\to 24\,h}$ in the rIL-2 + IM repeated doses and IM alone (repeated doses) groups was 1.54-fold and 1.36-fold higher compared with the IM alone (single dose) group, respectively ($P < 0.01$; < 0.05, respectively using Bailer's method). Furthermore, plasma $AUCs_{0\to 24\,h}$ in the rIL-2 + IM repeated doses group was 1.13-fold higher when (not significant) compared with the IM alone (repeated doses) group.

Finally, we observed the half-life prolongation increase in the rIL-2 + IM repeated dose group as compared with the IM repeated dose alone group.

Influence of rIL-2 pretreatment in P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) expression by western blot analysis in intestine (Figs 2 and 3)

Western blot analysis of ABCB1 and ABCG2 expression was done in three intestine segments of six mice from the IM alone repeated doses group and of six mice from the rIL-2 + IM repeated doses group (from the pharmacokinetic study).

We showed that ABCB1 expression seemed to be significantly lower in the rIL-2 + IM-treated group

compared with the IM alone group in the duodenum segment ($P < 0.01$, Mann–Whiteny test), jejunum segment ($P < 0.05$) and ileum segment ($P < 0.05$) (Fig. 2).

Considering the ABCG2 expression, it seemed to be significantly lower in the rIL-2 + IM-treated group as compared with the IM alone group in the duodenum segment ($P < 0.05$).

In contrast, rIL-2 failed to significantly decrease intestinal ABCG2 expression in the jejunum and ileum segments (Fig. 3).

Influence of imatinib orally administered twice a day (in the efficacy study) on intestinal (duodenum segment) ABCB1 and ABCG2 expression (Fig. 4)

The western blot analysis showed that ABCB1 expression was significantly increased in mice that received the IM treatment twice a day for 11 days compared with the control group.

On the contrary, the western blot analysis showed that ABCG2 expression was unchanged in mice that had received the IM treatment compared with the control group.

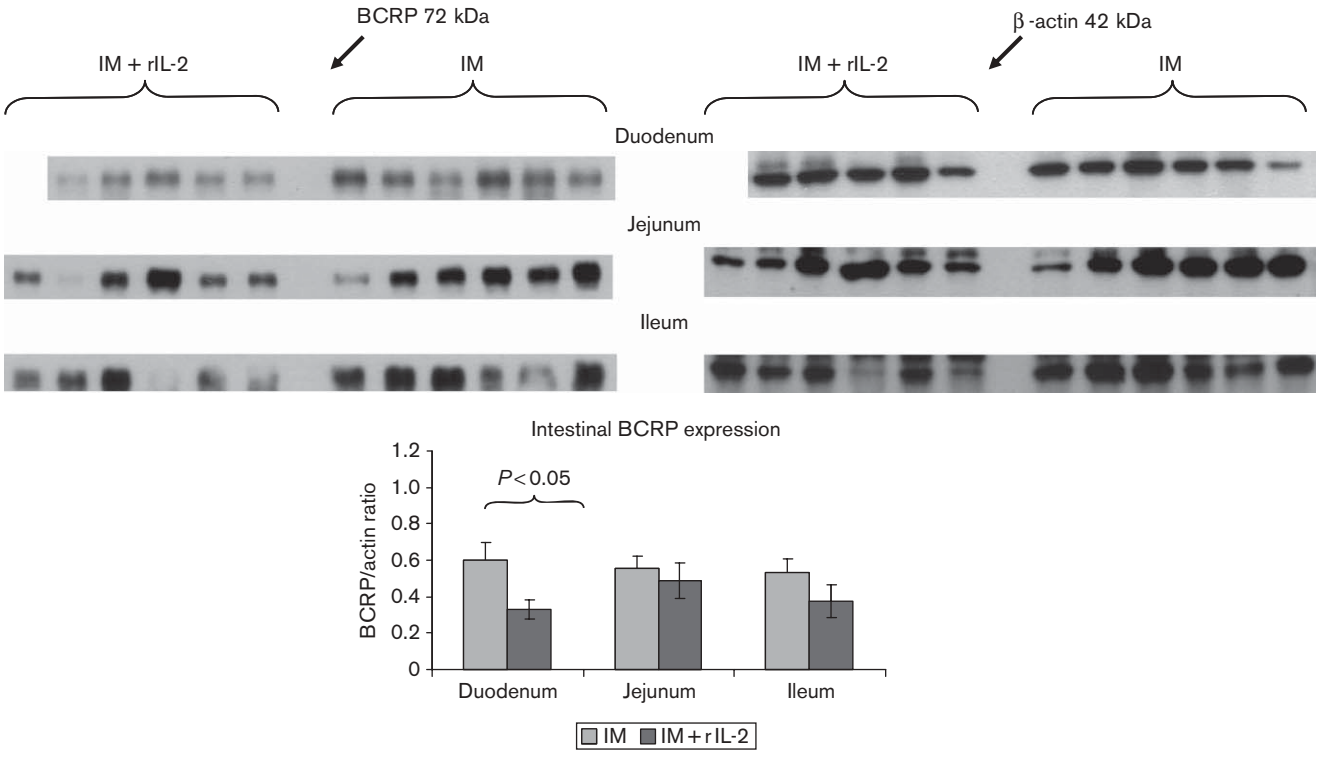
Discussion and conclusion

The principal objective of this study was to determine the pharmacokinetic consequences of combining rIL-2 with IM, given that the pharmacodynamic benefits of this combination have been established previously [1]. In this study, the pharmacokinetics of IM was investigated after single (group 1) or repeated (group 2) oral doses in mice. The effects of rIL-2 pretreatment on the plasma pharmacokinetics of IM after repeated oral administration (group 3) were also investigated in mice.

Comparison between groups 1 and 2

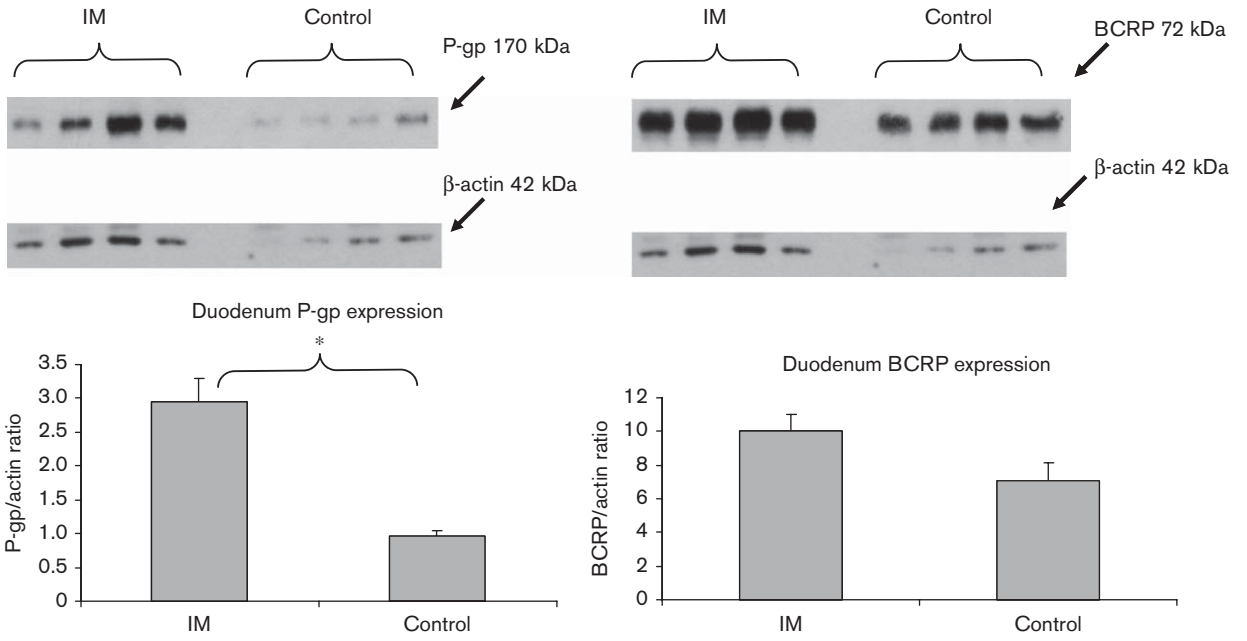
The pharmacokinetic study showed a significant IM AUC increase (and C_{max} increase) in group 2 as compared with group 1 (Table 1). This increase in AUC could be explained by either an inhibition of cytochrome P-450, or an increase of IM absorption caused by repeated oral administration. The IM elimination half-lives were comparable in both groups, suggesting that the increases in AUC and C_{max} are better explained by the increase of IM absorption. As IM is a substrate of both ABCB1 and ABCG2 [15,29], different absorption rates could arise from the inhibition of intestinal ABCB1 and/or ABCG2 functionality when IM was administered in repeated

Fig. 3



ABCG2 immunodetection from the intestine segments of mice treated by recombinant interleukin-2 (rIL-2) + imatinib (IM) or IM alone. The mean ABCG2/actin ratio ± mean standard error from six intestines of mice per group are represented in the histogram.

Fig. 4



ABCB1 and ABCG2 immunodetection from the duodenum segments of mice treated by imatinib (IM) alone (twice a day for 11 days) versus control (mice without treatment). The mean ABCB1 and ABCG2/actin ratio ± mean standard error from four intestines of mice per group are represented in the histogram. P-gp, P-glycoprotein. *ABCB1 expression in treated mice was significantly higher then in control mice ($P<0.05$).

doses. This hypothesis is consistent with an earlier report from Hamada *et al.*, which specifies that IM is both a substrate and an inhibitor of P-gp functionality. Indeed, using a calcein-AM assay, Hamada *et al.* estimated the K_i values for the inhibition of P-gp function by cyclosporine A and IM to be 6.1 and 18.3 µmol/l, respectively. In another study, functional inhibition of P-gp was observed at an IM concentration of 5 µmol/l (or 2949 ng/ml) [29]. Given that up to the 16-h time point, the plasma concentration of IM in our study ranged from 2400 to 2900 ng/ml in both groups, we can reasonably suppose that 24 h after an initial IM administration (in group 2), intestinal P-gp functionality should be attenuated relative to group 1. Thus, the inhibition of intestinal P-gp activity because of repeated IM administration in group 2 could increase IM C_{max} and AUC as compared with group 1.

A western blot analysis of ABCB1 expression in duodenum segments from IM-treated mice (150 mg/kg, twice a day for 11 days) revealed a significant induction of this transporter as compared with the control group (Fig. 4). These results agree with those obtained *in vitro* by Burger *et al.* [19], which indicated that chronic exposure to IM-induced expression of ABCB1 and ABCG2 in Caco-2 cells. Interestingly, other studies have shown that 5 days of treatment with cyclosporine A (a well-known inhibitor of P-gp function) increased P-gp expression in rat liver [30]. In addition, cyclosporine-induced renal expression of P-gp has also been observed in humans [31]. These observations collectively suggest the following hypothesis: IM first inhibits P-gp functionality, which would explain the increases in IM C_{max} and AUC in group 2 as compared with group 1. Decreased P-gp functionality could then trigger an increase P-gp expression, as has been observed in rats treated with cyclosporine [30].

Despite its high oral bioavailability in humans (98%), the pharmacokinetic profile of IM varies significantly from patient to patient [15]. Furthermore, the oral bioavailability of IM has been found to range from 48 to 70% in mice [32]. This suggests that pharmacokinetic resistance to IM could be developed especially in the context of chronic IM treatment – through modulation of ABCB1 and/or ABCG2 expression or function in the intestinal tract, with a subsequent decrease in IM oral bioavailability. Indeed, a study has shown that orally administered elacridar and pantoprazole significantly increased the AUC of orally administered IM in wild-type mice by inhibiting ABCB1 and ABCG2, respectively [32].

Comparison between groups 2 and 3

In C57B1/6 mice, treatment with rIL-2 was found to increase the oral bioavailability of paclitaxel (an ABCB1 substrate) by decreasing the intestinal expression of

ABCB1; the metabolism of paclitaxel was not significantly impacted. This is noteworthy, as IM, paclitaxel is a well-known substrate of human CYP 3A4 and 2 C8 [33].

To determine whether the rIL-2 + IM combination might hold pharmacokinetic as well as pharmacodynamic benefits, the plasma concentration of IM was monitored over time, after repeated administration of IM alone (group 2) or in combination with rIL-2 (group 3). This pharmacokinetic study revealed an increase in plasma IM $AUC_{0\rightarrow 24\,h}$, with a statistically significant increase in C_{max}, in group 3 as compared with group 2 (Table 1). The statistically insignificant IM $AUC_{0\rightarrow 24\,h}$ increase in group 3 as compared with group 2 (in contrast to the significant increases in C_{max} and $C_{24\,h}$) could be explained by the high interindividual variability in IM concentrations in mice corresponding to time points other than C_{max} and $C_{24\,h}$.

These pharmacokinetic trends could be related to a modification of oral IM absorption due to rIL-2. This hypothesis is further supported by the ABCB1 and ABCG2 expression study, which revealed a significant decrease of ABCB1 expression in three murine intestinal segments (duodenum, jejunum and ileum) in group 3 as compared with group 2 (Fig. 2).

Furthermore, the western blot analysis reveals a significantly decreased expression of ABCG2 in duodenum segments from group 3 as compared with group 2 (Fig. 3). Thus, group 3 could exhibit the combined effects of P-gp function inhibition by IM due to repeated administration, and a decrease in P-gp expression due to rIL-2. By decreasing ABCG2 expression in the duodenum and ABCB1 expression in the duodenum, jejunum and ileum, rIL-2 could have decreased IM efflux through ABCB1 and/or ABCG2 in the intestinal tract, and consequently increased the oral absorption and C_{max} of IM. In contrast, IM is also a substrate of other drug-uptake transporters, including the human organic cation transporter (hOCT1); such transporters are also important in determining the clinical response to IM [34]. Indeed, patients with high hOCT1 expression had a superior complete cytogenetic response to IM. Modifications in the expression or function of this protein could produce increases in the C_{max} or AUC of IM.

In addition, a significant increase in the IM trough concentration was observed in group 3 as compared with group 2 (Table 1). These pharmacokinetic modulations of IM due to rIL-2 treatment could be very interesting in light of pharmacodynamic considerations. Indeed, a study has shown that the IM concentration required to abolish cell lines positive for Bcr-Abl *in vitro* is 1 μmol/l, equal to 570 ng/ml [35]. A clinical pharmacokinetic/pharmacodynamic study has provided evidence that AUC, C_{max}, trough level and duration above a concentration of 570 ng/ml strongly correlate to the initial pharmacodynamic effects of IM. This is not surprising, as these measures of exposure are directly linked to the dose of IM [17]. In addition, IM is well tolerated, and C_{max} is more strongly associated with higher efficacy rather than higher toxicity [17,36]. Our pharmacokinetic study has also shown that the trough concentration ($C_{24\,h}$) in group 3 (615 ± 37 ng/ml) exceeded 570 ng/ml, and was significantly higher than in group 2 (146 ± 57 ng/ml). This result means that rIL-2 allowed the mean IM plasma concentration to remain above 570 ng/ml in mice. In addition, it has been proven in humans that the steady state trough plasma concentration of IM is a significant predictor of complete response in chronic-phase myeloid leukaemia [35]. Another study has shown that, among patients with a plasma IM trough concentration of 490 ± 120 ng/ml, the complete cytogenetic response was 75.9%, whereas in patients with an IM trough concentration of 889 ±148 ng/ml, the complete cytogenetic response percentage rose to 85.4% (P=0.01) [37]. The significant increase of the IM trough concentration ($C_{24\,h}$) in group 3, as compared with group 2, could result from the inhibition of ABCB1 and/or ABCG2 transport by rIL-2, and thus to a reduced rate of IM elimination through the renal and/or biliary canaliculi. Such a mechanism does not exclude potentially significant interactions between cytochrome P-450 and rIL-2.

The selected dosage regimen for rIL-2 (0.16 MUI, injected twice a day for 3 days) has been previously determined to decrease intestinal P-gp in mice, and to closely resemble a typical human dosage. In addition, this rIL-2 dosage has showed anticancer activity in a murine tumour model (Lewis lung carcinoma model) [38]. The dose schedule for IM (150 mg/kg, once a day) also corresponds to a typical regimen in humans. Thus, by using rIL-2 in combination with IM, we could simultaneously draw upon the anticancer activity of rIL-2, a significantly increased IM C_{max}, and plasma IM trough concentration ($C_{24\,h}$) of over 570 ng/ml in mice. These combined pharmacodynamic and pharmacokinetic considerations could avoid or attenuate the pharmacokinetic resistance to IM caused by subtherapeutic IM concentrations.

The mechanism by which cytokines modulate P-gp expression and activity is still not fully understood. The immune system is regulated by a very complicated network of cytokines and cytokine receptors. Transcriptional activation of cytokine genes is controlled by specific transcription factors, such as NF-κB, STAT and NF-AT [39]. Using the HCT-15 human colon carcinoma cell line, Bentires-Alj *et al.* [40] have showed that NF-κB is involved in the regulation of MDR1 mRNA expression during the onset of drug resistance. In particular, a reduction in MDR1 mRNA and P-gp expression was observed upon inhibition of NF-κB. Knowing that these transduction pathways are strongly conserved between species, it seems reasonable to assume that interactions observed between P-gp and cytokines in model organisms could be extrapolated to humans.

Considering the potential development of pharmacokinetic resistance to IM through physiological ABCB1 and ABCG2 expression and/or modulation of function, it would be interesting to further investigate IM efficacy and toxicity when combined with rIL-2 treatment for clinical applications.

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