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Report

Recombinant interleukin-2 treatment decreases P-glycoprotein activity and paclitaxel metabolism in mice

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Recombinant rIL-2 was reported to be able to decrease P-glycoprotein (P-gp) expression in cultured cells from human colon carcinoma. P-gp is considered an important factor in the control of Taxol® efflux from tumor cells. Based on the premise that Taxol pharmacokinetic parameters could be modified as a result of diminished P-gp expression induced by recombinant interleukin (rIL)-2 and that this might elicit an interaction between the two drugs, we evaluated the pharmacokinetics of a novel strategy combining i.p. immunotherapy with rIL-2 and a cytotoxic agent, Taxol. Mice were allocated to two groups treated with rIL-2 (15 μg $\times$ 2/day from day 1 to 4) then Taxol (10 mg/kg i.p. day 5) or Taxol (10 mg/kg i.p.) alone (control group). The Taxol + rIL-2 combination provoked the development of ascites, presumably due to the presence of Cremophor EL in the Taxol preparation. Paclitaxel was measured in plasma and ascites by HPLC with UV detection. Paclitaxel pharmacokinetics were strongly modified by rIL-2 pretreatment. Compared to that observed in control mice, the apparent volume of distribution increased dramatically (V_d/F = 18.2 versus 4.1 l/kg) and the apparent plasma clearance decreased (Cl/F = 1.12 versus 1.66 l/h/kg). P-gp expression was determined in the liver, lung, intestine, brain and kidney in the two groups by immunodetection with the C219 anti-P-gp monoclonal antibody. A significant decrease in P-gp expression was observed in the intestine and in the brain in the rIL-2-pretreated mice as compared to controls. To study the functionality of P-gp, we compared digoxin (a model P-gp substrate) pharmacokinetics before and after pretreatment with rIL-2 (10 μg $\times$ 2/day from day 1 to 4), after a single 1 μg oral dose of digoxin used to quantify P-gp activity. Results showed a decrease in oral digoxin clearance after rIL-2 pretreatment indicating modified P-gp activity. We conclude that rIL-2 pretreatment is able to decrease P-gp activity and paclitaxel metabolism *in vivo*. This is the first study to demonstrate a decrease in P-gp activity and expression in organs such as the brain *in vivo*. A novel strategy combining immunotherapy with rIL-2 and a cytotoxic agent could potentially improve clinical results, particularly in brain cancer. [© 2002 Lippincott Williams & Wilkins.]

Key words: Digoxin, mice, P-glycoprotein, paclitaxel, pharmacokinetics, rIL-2.

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Introduction

P-glycoprotein (P-gp) is an ATP-dependent, efflux membrane transporter with broad substrate specificity for a number of structurally diverse drugs. The *MDR1* (multidrug resistance) gene encodes human P-gp. In mice, two P-gps (encoded by the *mdr1a* and *mdr1b* genes) appear to perform the function of the single human protein. The mouse *mdr1a* gene is predominantly expressed in the intestine, liver and capillary endothelial cells of the brain and testis, whereas the *mdr1b* gene is predominantly expressed in the adrenal, placenta, ovary and pregnant uterus. Similar levels of *mdr1a* and *mdr1b* gene expression are found in the kidney. Apical expression of P-gp in tissues results in reduced drug absorption (gastrointestinal tract), and enhanced elimination of drugs into the bile (liver) and the urine. P-gp is therefore an important underlying mechanism of drug interactions in animals and humans, and decreasing its expression could be a key to enhanced drug activity.

A wide variety of anti-neoplastic agents such as anthracyclines, vinca alkaloids and the taxane, paclitaxel, are P-gp substrates. Overexpression of P-gp leads to the *MDR* phenotype, which simultaneously renders tumor cells resistant to several drugs.

Recombinant interleukin (rIL)-2 is instrumental in mounting an immune response: it stimulates NK cell

and cytotoxic T lymphocyte activity. Moreover, P-gp, which is expressed in the plasma membrane of normal human T lymphocytes, is also involved in the transport of IL-2.[1]

We studied the impact of rIL-2 on the pharmacokinetics of paclitaxel for several reasons. Firstly, paclitaxel is a substrate for P-gp known to be involved in its mechanism of resistance, although this is not the only one.[2] Secondly, rIL-2 has been shown to decrease mdr1 mRNA as well as P-gp expression in cultured cells from human colon carcinoma.[3]

Sparreboom *et al*.[4] have shown that P-gp limits the bioavailability of paclitaxel after oral administration and mediates the direct excretion of the drug from the systemic circulation into the intestinal lumen.

Intestinal P-gp also contributes to the elimination of parenterally administered substrate drugs by direct secretion of the drug into the intestinal lumen. Accordingly, rIL-2 could modify paclitaxel pharmacokinetics given its alleged effects on P-gp expression *in vivo*.

The aim of this study was to investigate with a mouse model, the pharmacokinetics of a potentially novel strategy combining i.p. rIL-2 immunotherapy with paclitaxel based on the premise that rIL-2 is able to modify P-gp expression (and activity) *in vivo*. P-gp expression was investigated by Western blot analysis in mouse tissues. The effect of rIL-2 on P-gp activity was investigated *in vivo* by studying digoxin pharmacokinetics.[5,6]

Materials and methods

Chemicals

Paclitaxel was supplied by ICN Pharmaceuticals (Orsay, France), docetaxel (the internal standard) by Bellon Rhône Poulenc Rorer (Montrouge, France) and Taxol® was supplied by Bristol-Myers Squibb (Puteaux, France).

Methanol and absolute ethanol were obtained from Merck (Nogent sur Marne, France), and acetonitrile, ammonium acetate, triethylamine, *n*-hexane and 1-ml cyano Bond Elut columns were obtained from Prolabo (Fontenay sous Bois, France).

Digoxin Nativelle was supplied by Procter and Gamble Pharmaceuticals (Neuilly sur Seine, France.)

Animals

Adult Swiss male mice (12 weeks of age) with a mean body weight of 30 g were purchased from Charles River (St Aubin les Elbeuf, France). Animals were housed in cages with food and water *ad libidum*. Animals were acclimated for 1 week prior to the start of experiments.

Pharmacokinetic study of paclitaxel

Taxol®, paclitaxel formulated in Cremophor EL and ethanol (1:1, v/v), was diluted with isotonic sodium chloride to a final concentration of 2 mg/ml. Two groups of mice were compared. In the first group, mice received rIL-2 (15 μg/day from day 1 to 4) twice daily (at 9.30 a.m. and 5.30 p.m.) then Taxol (10 mg/kg i.p. day 5). In the second group, mice received Taxol (10 mg/kg i.p.) alone.

Blood samples were drawn on sodium heparin at 15, 30, 60 and 90 min, and 2, 3, 5, 7 and 24 h after administration, using six mice per time point. Plasma was separated by centrifugation (20 min at 1500 *g*), delivered in three aliquots and stored at -20°C until analysis.

Paclitaxel assay

Plasma paclitaxel concentrations were determined by HPLC after solid-liquid extraction using a modified version of the Willey *et al*. method.[7] In brief, 100 μl of plasma was mixed with 100 μl of ammonium acetate 0.2 M and 50 μl of docetaxel used as the internal standard for 20 s. Paclitaxel was extracted from plasma by solid-phase extraction onto 1-ml cyano Bond Elut columns. The columns were first conditioned with 1 ml of methanol, then with 1 ml of 0.01 M ammonium acetate. The samples were loaded onto Bond Elut columns, and washed with 2 ml of 0.01 M ammonium acetate, 2 ml of 20% methanol in 0.01 M ammonium acetate and 1 ml of *n*-hexane. The columns were dried under vacuum for 1 min. Paclitaxel and the internal standard were eluted using 2 ml of 0.1% triethylamine in acetonitrile.

The eluents were evaporated under nitrogen at 30°C. The residues were reconstituted in 100 μl of a water:acetonitrile mixture (55:45 v/v) and vortexed for 30 s. Aliquots of 50 μl of each sample were injected onto the HPLC column.

For the analysis of ascites, paclitaxel was extracted by protein precipitation with acetonitrile. Then 100 μl of ascites was mixed with 200 μl of acetonitrile and 50 μl of the internal standard (docetaxel). After centrifugation, 50 μl of supernatant was injected onto the HPLC column.

The chromatographic system consisted of a Shimadzu LC6A pump, a Shimadzu SPD6A detector and

a Shimadzu CR5A recorder (Touzard et Matignon, Les Ulis, France). Reverse-phase HPLC was performed using a Nucleosil C18 column (250 × 4.6 mm; 5 μm). The mobile phase, a water:acetonitrile mixture (55:45 v/v), was delivered at a flow rate of 1.5 ml/min and UV detection was set at 227 nm. Intra-day and inter-day variation coefficients of paclitaxel were below 10%, and ranged between 0.1 and 5 μg/ml. The quantification limit was 0.05 μg/ml.

rIL-2 assay

Serum concentrations of residual rIL 2 were measured in six mice per time point with an ELISA (Immunotech). The sensitivity of the assay in plasma is 5 pg/ml. Blood samples were collected on sodium heparin at 16, 18 and 40 h after the last injection of rIL-2 for determination of residual rIL-2.

Digoxin pharmacokinetics

The experiment was carried out with 54 male mice divided into nine groups of six mice. Each group corresponded to one of the nine plasma sampling times (15 and 30 min, and 1, 2, 3, 4, 6, 8 and 24 h). Before the experiment, the mice were left to fast 18 h with water *ad libidum.* They were then fed 20 μl of digoxin solution (5 μg/0.1 ml) corresponding to a total dose of 1 μg, i.e. 0.03 mg/kg of digoxin. Blood samples were collected and processed as detailed above. After a 'wash-out' period of 3 weeks, mice were given 10 μg/kg rIL-2 i.p., twice a day for 4 days. After an 18-h fasting period, digoxin pharmacokinetics were reinvestigated as already described.

Plasma digoxin concentrations were determined by the AxSym Digoxin II assay which is a microparticle enzyme immunoassay (MEIA; Abbott, Rungis France). All plasma samples were assayed together with calibration and quality control.

The inter- and intra-day coefficients of variation of plasma levels were below 3% for a 2 ng/ml concentration. The lower quantification limit was 0.3 ng/ml.

Pharmacokinetic analysis

Plasma concentration–time data were analyzed according to a compartment-independent approach. The pharmacokinetic parameters were also obtained from non-linear curve fitting using a one-compartment model with first-order absorption, K_a, and elimination, k_{10} (Micropharm Pharm Research).[8] The K_a and k_{10} estimates were used for calculation and extrapolation purposes in the compartment-independent approach.

The area under the plasma concentration–time curve (AUC) and the area under the moment curve (AUMC) were calculated from zero to infinity using the trapezoidal rule. The apparent plasma clearance (CL/*F*) and apparent volume of distribution (V_d/*F*) were then determined from the equations:

$$CL/F = dose/AUC_{0-\infty}$$

$$V_d/F = dose/AUC_{0-\infty} \times (AUMC_{0-\infty}/AUC_{0-\infty} - 1/K_a)$$

Histological examination of livers

Livers were fixed in Bouin's liquid and embedded in paraffin. The slides were stained with HES (hematoxylin & eosin safran).

Determination of P-gp protein expression by Western blot analysis

Mice were sacrificed 24 h after the last rIL-2 injection and samples were scraped from the jejuno-ileal mucosa that had been placed on ice.

Mucosa. samples from two mice were pooled 3 times. The same procedure was undertaken for mice administered Taxol alone (control group). Samples from other organs (brain, lungs, kidneys and liver) of two mice were also pooled.

Crude membranes from all tissues were prepared according to the method of Jetté.[9] Samples of 10 μg of membrane proteins were separated on acrylamide gel (7.5%) electrophoresis.[10]

P-gp was immunodetected after transfer onto nitrocellulose membrane using anti-P-gp C219 monoclonal antibody (Dako, Carpenteria, CA) diluted 200-fold in 10 mM phosphate buffered saline and Tween 20 (0.05%).

Immunoblots were performed with enhanced chemiluminescence (ECL) reagents (Amersham, Little Chalfout, UK) according to the manufacturer's instructions.

The band image was taken with a CCD camera and band intensity was estimated with Kodak digital analysis ID software.

Statistical analysis

Statistical analyses were performed with the ANOVA test using the Stat View program for comparisons of

immunoblot density. Digoxin pharmacokinetic parameters (without and with pretreatment with rIL-2) were compared with the paired *t*-test.

Results

Paclitaxel pharmacokinetics

As shown in Figure 1, plasma paclitaxel concentrations were lower in the rIL-2-treated group than in the control group.

As ascites were constantly observed in the rIL-2-pretreated mice, ascites paclitaxel concentrations were also measured. Paclitaxel concentrations were higher in ascites than in plasma and decreased from 140–120 μg/ml at 0.25–1.50 h to 25–20 μg/ml at 5–7 h.

The compartment-independent and -dependent approaches provided very close pharmacokinetic estimates for CL/*F* and V_d/F. As shown in Table 1, rIL-2 pretreatment caused a drop in the mean CL/*F* and a dramatic increase in the mean V_d/F.

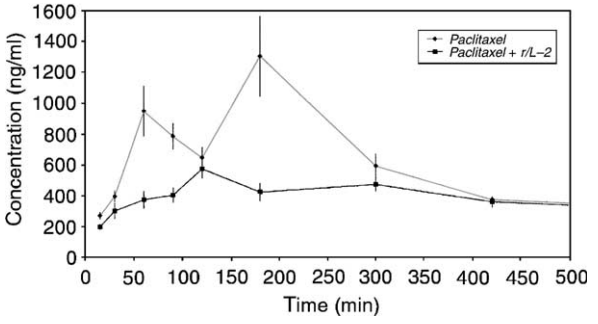

Figure 1. rIL-2 effects on paclitaxel pharmacokinetics administration in mice following i.p. administration.

Table 1. rIL-2 effects on paclitaxel pharmacokinetic parameters in mice (i.p. administration of both drugs)

Parameter	Controls	rIL-2-pretreated mice
C_{max} (μg/ml)	1.303	0.573
T_{max} (h)	2	3
CL/*F* (l/h/kg)	1.66	1.12
V_d/F (l/kg)	4.1	18.2

Pharmacokinetic parameters are derived from the compartment-independent method.

In order to understand why ascites developed in rIL-2-pretreated mice, we measured the volume of ascites obtained in three groups of five mice receiving, respectively, an i.p. injection of rIL-2+ Taxol, rIL-2 + glucose) or rIL-2 + Cremophor EL.

Results on day 5 showed that ascites were not induced by rIL-2 alone. The injection of rIL-2+ Cremophor EL was even more active in producing ascites than that of rIL-2 + Taxol (Table 2).

To understand the contribution of ascites to the paclitaxel V_d/F value in IL-2 pretreated mice, paclitaxel pharmacokinetics in ascites were analyzed and the corresponding AUC was estimated, 561 000 μg/l h. According to classic concepts, the V_d relative to plasma drug concentration is a function of drug distribution in the body:

$$V_d = V_P + C_T/C_P \times V_T$$

where V_P, V_T and C_P, C_T are the plasma and tissue volumes, and plasma and tissue concentrations, respectively. In the presence of ascites, the V_d value is then:

$$V_d = V_P + C_T/C_P \times V_T + C_{Tascites}/C_P \times V_{ascites}$$

in which the ratio $C_{ascites}/C_P$ is estimated by the ratio $AUC_{ascites}/AUC_P$, 62.7. Given the volume of ascites, 580 μl/30 g, the paclitaxel concentration in ascites contributed 1.21 l/kg to the total paclitaxel V_d.

Digoxin pharmacokinetics

Table 3 shows that higher plasma digoxin concentrations were observed in the rIL-2-treated group as compared to control group (Figure 2). In rIL-2-pretreated mice, C_{max} was 27.6 ng/ml versus 18.1 ng/ml in controls. The time to C_{max} was 0.5 h in both cases. At the 15 min and 1 h time points, the plasma digoxin concentration in the group pretreated with rIL-2 was 1.8-fold higher than in controls.

Again, the compartment-independent and -dependent approaches provided very close pharmacokinetic estimates for CL/*F* and V_d/F. As shown in Table 3, rIL-2 pretreatment caused a dramatic decline in CL/*F* (50%), but V_d/F remained unaffected.

Table 2. Ascites volume (μl)

	rIL-2 + Taxol	rIL-2 + glucose	rIL-2 + Cremophor EL
Ascites volume mean (n = 5)	580	130	870

Table 3. rIL-2 effects on digoxin pharmacokinetics in mice (oral administration of digoxin and i.p. administration of IL-2)

Parameter	Controls	rIL-2-pretreated mice
C_{max} (μg/ml)	18.1	27.6
T_{max} (h)	0.5	0.5
CL/*F* (l/h/kg)	14.3	7.75
V_d/*F* (l/kg)	52	43

Pharmacokinetic parameters are derived from the compartment-independent method.

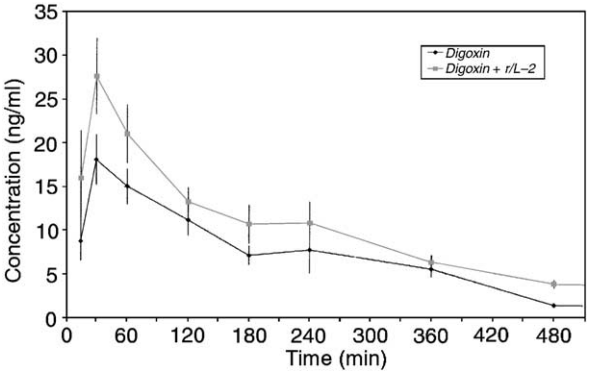

Figure 2. rIL-2 effects on digoxin pharmacokinetics in mice (oral administration for digoxin and i.v. administration for rIL-2).

IL-2 plasma concentrations

Mean residual plasma rIL-2 levels were 30, 17 and 1.8 pg/ml. at 16, 18 and 40 h after the last rIL-2 injection. The 16 h time point corresponded to Taxol administration.

Histological analysis of the liver

The liver was normal after 120 min in mice that received Taxol. After 24 h, discrete anisonucleosis was observed in hepatocytes in two out of three cases.

In mice that received Taxol and rIL-2, a small infiltrate of polynuclear cells was found around the portal spaces in two out of three cases after 120 min. Hepatocytes were normal. After 24 h the inflammatory infiltrate persisted in two out of three cases.

P-gp expression by Western blot analysis

Immunoblots showed a significant decrease in P-gp protein expression in the intestine (immunoblot densitometry, relative optical density (OD): 36.9 ± 1.1 versus 14.6 ± 4.4 for the control group and Taxol+IL-2 group, respectively, $p = 0.002$) and in the brain (OD, 38.7 ± 0.6 versus 13.7 ± 4.0 for the control group and rIL-2 groups, respectively). No statistical difference was observed in immunoblot density for the kidneys, liver and lungs (Figure 3).

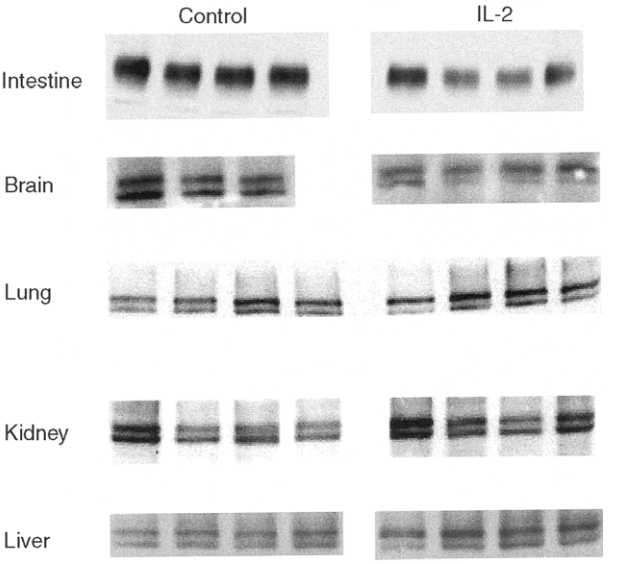

Figure 3. Immunodetection of P-gp from organs of mice treated with Taxol alone (Control) and of mice treated with rIL-2 then Taxol (IL-2).

Discussion

Our results show that rIL-2 increased the AUC of paclitaxel administered i. p. as attested by a decrease in the apparent clearance (−30%) and an increase in the apparent volume of distribution (+350%). These modifications were associated with decreased P-gp expression in brain and intestine tissues, and reduced functionality of P-gp.

Intraperitoneal administration of chemotherapy is an interesting approach for malignancies that remain confined to the peritoneal cavity. Because of certain properties, i.e. its activity in ovarian cancer, its hepatic metabolism and a lack of vesicant reactions, paclitaxel is a candidate for i.p. administration.[11–13]

Taxol+IL-2-pretreated mice developed ascites. The combination of Cremophor EL+IL-2 seems to have been the cause of this adverse effect. The effect of the paclitaxel dose sequestered in ascites on the increase in the volume of distribution was negligible (1.2 relative to a 16 l/kg increase), indicating that rIL-2 pretreatment did indeed induce an increase in paclitaxel tissue distribution.

Additionally, ascites could be viewed as a drug reservoir allowing a slow and sustained release of paclitaxel in nearby tissues and plasma, which would explain the smoother pharmacokinetic profile in rIL-2-treated mice compared to control mice (see Figure 1).

The increase in the volume of distribution of Paclitaxel upon injection into rIL-2-pretreated mice may have been due to rIL-2 inhibition of P-gp-promoted efflux of paclitaxel from organs mediated by underexpression of the protein and a possible decrease in P-gp activity *in vivo*. Following rIL-2 administration, P-gp expression was found to be significantly reduced in intestine and in brain tissue while no difference was observed in lungs, kidneys and liver. The mouse intestine and brain are organs where the *mdr1a* gene is predominantly expressed compared with other *mdr* genes.[14,15] How rIL-2 specifically regulates the *mdr1a* gene is a subject that merits investigation.

Paclitaxel is extensively metabolized by cytochromes P-450 (2C8 and 3A4).[16–18] According to Sparreboom[19] after a single i.v. bolus of 20 mg/kg in mice, 3′*p*-hydroxy paclitaxel and 6-hydroxypaclitaxel were produced. These metabolites were not detectable in plasma nor in any of the other tissues, indicating that the major part of these metabolites was probably excreted into the bile without significant reabsorption from the intestinal lumen. The amount of these metabolites excreted in the feces accounted for about 25% of the administered dose. The decrease in the CL/*F* could be due to the impact of IL-2 inhibiting properties on cytochromes P450.[20–23]

Digoxin is transported by the ATP-dependent efflux pump P-gp. In mice, digoxin pharmacokinetics is mainly linked to P-gp activity (excretion of digoxin by P-gp expressed in the membrane of renal and intestinal cells) and poorly associated with other metabolization and elimination factors. Digoxin is not extensively metabolized. The comparison of plasma digoxin concentrations and experimental pharmacokinetic parameters with or without rIL-2 pretreatment will enable us to evaluate a possible rIL-2 inhibiting effect on P-gp activity.

Elevated plasma digoxin concentrations with a 1.8-fold higher C_{max} have been observed after administration of rIL-2 in mice. Digoxin is primarily eliminated by the kidneys, and the mechanism of renal clearance involves glomerular filtration, tubular secretion and reabsorption. Renal tubular secretion of digoxin is mediated by P-gp.

Thus rIL-2 inhibition of P-gp-mediated digoxin absorption and elimination may be a common mechanism leading to elevated digoxin concentrations. In previous experiments. Schinkel *et al*. reported on *mdrla*(−/−) knockout mice lacking the mouse *mdrla* P-gp. They showed markedly higher plasma concentrations (2-fold higher) of digoxin 4 h after injection of 1 mg/kg with slower elimination compared to that observed in wild-type animals.[24,25] In mice, *mdr1a* is the predominant isoform for digoxin transport. Kawahara *et al*. reported that after delivery of 1 mg/kg i.v. in *mdr1a*(+/+) and *mdr1a*(−/−) mice, total clearance of digoxin was decreased to 30% of that found in *mdr1a*(+/+) mice.[26]

In our study, pretreatment of mice with rIL-2 resulted in a significant decrease in plasma digoxin CL/*F* illustrated by higher plasma concentrations and a slower elimination half-life compared to that observed in control mice. The Western blot analysis of P-gp showed a 2.5-fold decrease in intestinal P-gp expression and a 2.8-fold decrease in brain P-gp expression after pretreatment with rIL-2, indicating that P-gp expression and activity were modified by pretreatment with rIL-2.

This is the first study to demonstrate a decrease in P-gp expression in the intestine and the brain of mice, and modified P-gp activity after the administration of rIL-2. A novel strategy combining immunotherapy with a cytokine such as rIL-2[27,28] and a cytotoxic agent could potentially improve clinical results, particularly in brain cancer.

Moreover, P-gp is also involved in resistance to paclitaxel and rIL-2 could be used to decrease the efflux of anticancer drugs from some organs.

In conclusion, the modifications of the pharmacokinetic parameters induced by rIL-2 pretreatment have been shown to enhance tissue distribution and to lower paclitaxel metabolism: these changes were associated with modified P-gp expression and activity. rIL-2 immunotherapy combined with a cytotoxic agent could potentially improve clinical results and particularly when the blood–brain barrier has to be crossed to target both primary and metastatic intracranial tumors. P-gp is also involved in resistance to paclitaxel and rIL-2 could decrease the extrusion of anticancer drugs from some organs. Proven MDR P-gp+tumors could be enticed into responding to chemotherapy combined with IL-2.[29]

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