Recombinant interleukin-2 pre-treatment increases anti-tumor response to paclitaxel by affecting lung P-glycoprotein expression on the Lewis lung carcinoma

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The aim of the present study was to examine modifications of anti-tumor activity and toxicity of paclitaxel (PLX) when given p.o. after recombinant interleukin-2 (rIL-2) to Lewis lung carcinoma-bearing mice. PLX was given orally to mice at the dose of 15 mg/kg on day 8 and 30 mg/kg on day 15, either alone or after 16.5 μg of rIL-2 given i.p. twice a day either 1 or 3 days before. The anti-tumor activity was higher and PLX hematological toxicity not increased if orally administered PLX was given after a 3-day rIL-2 pre-treatment rather than if given alone. Lung metastasis was significantly lower and s.c. tumors were smaller in the PLX + rIL-2 group than in the PLX or rIL-2 or non-treated groups. In addition, a decrease in lung P-glycoprotein expression (investigated by Western blot analysis) was observed 1 h after the last administration of rIL-2 on day 7. *Anti-Cancer Drugs* **17:195–199 © 2006 Lippincott Williams & Wilkins.**

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Introduction

Paclitaxel (PLX), a drug from the tubulin-inhibiting taxane family, is used for the treatment of breast, ovarian and non-small cell lung carcinoma [1,2]. Following its administration, PLX undergoes extensive metabolism and biliary excretion. Three main metabolites are formed via cytochrome CYP2C8- and CYP3A4-mediated pathways. Its main side-effect is a reversible hematotoxicity [3,4]. PLX is a known substrate of P-glycoprotein (P-gp) and basal expression of P-gp plays a role in the resistance of cancer cells to PLX by its action as an efflux pump [5,6].

Physiologically, P-gp protects the body from toxicity caused by xenobiotics or endogenous substances by preventing their absorption from the intestinal tract and their distribution into specific organs, and by promoting their clearance by its action in the bile canaliculi and the kidneys. These different roles may explain the results obtained from clinical trials investigating the enhanced bioavailability of orally administered PLX combined with a P-gp inhibitor such as cyclosporine, MS-209 (a quinoline type reversal agent) or zosuquidar trihydrochloride [7,8].

The multidrug resistance (*MDR1*) gene encoding human multidrug resistance-related P-gp may play an important role in the resistance of lung cancer [9]. Recombinant interleukin-2 (rIL-2) has been shown to decrease *MDR1* mRNA as well as P-gp expression in cultured cells from human colon carcinoma [10], and *in vivo* both in the intestine and the brain of Swiss mice [11]. This cytokine has many effects on the body, particularly by activating the immune cells [12] and by inducing cancer cell destruction [13,14]. We have also reported that a 3-day pre-treatment by rIL-2 increases PLX oral bioavailability in mice [15].

Here, we studied the anti-tumor activity and toxicity of PLX given orally after i.p. rIL-2 pre-treatment in Lewis lung carcinoma (LCC)-bearing mice. We evaluated the effects of PLX alone, rIL-2 alone and their association on the growth of s.c. transplanted LLC tumors and on the number of spontaneously arising LLC lung metastases. We also measured P-gp expression in the mouse lung after pre-treatment with rIL-2.

Materials and methods

Tumor cells

Cell culture media and reagents were purchased from Invitrogen Life Technologies (Cergy Pontoise, France). LLC cells were kept frozen in 90% FBS/10% DMSO (the cells were supplied by UMR 8121, Gustave Roussy

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Institute). They were cultured for 10 days in RPMI 1640 medium glutamate supplemented with 10% heat-inactivated FBS and a 1× mixture of antibiotics (sodium penicillin G 10 000 IU/ml and streptomycin sulfate 10 000 μg/ml).

Drug preparation

PLX was prepared from a Taxol vial (Laboratory BMS, Puteaux, France) which contains 30 mg/5 ml with Cremophor-EL and ethanol. Either 10 or 2.5 ml sterile saline was added to obtain, respectively, a 2 or 4 mg/ml solution [3]. An aliquot of 150 μl of these solutions was administered by force-feeding to mice (average weight 20 g); respectively, 15 and 30 mg/kg/dose.

rIL-2 (Proleukin; Chiron, Suresnes, France) containing 18×10^6 IU was reconstituted with 1.2 ml of water. Then, 1 ml of the reconstituted preparation was diluted with 9 ml of 5% dextrose to obtain a final solution of 1.8×10^6 IU/ml. Aliquots of 150 μl were injected i.p. twice a day for 1 or 3 days (i.e. 16.5 μg of rIL-2 by injection) [3].

In-vivo protocol

Experiments were carried out on 6- to 8-week-old female C57Bl/6 mice (Charles River, L' Arbresle, France), which were given water and food *ad libitum*, in accordance with European Community guidelines.

LLC cells (10^6) were injected on day 0 in a volume of 0.2 ml of sterile saline into the back of 50 mice. On day 5, when tumors reached a volume of 30–60 mm^3, mice were randomized in five groups of 10 mice each: control (Group 1), rIL-2 alone (16.5 μg twice daily from day 5 to 7 and from day 12 to 14) (Group 2), PLX alone (15 mg/kg on day 8 and 30 mg/kg on day 15) (Group 3), rIL-2 twice daily on days 7 and 14, and then PLX 15 mg/kg on day 8 and 30 mg/kg on day 15 (Group 4), and rIL-2 twice daily from day 5 to 7 and from day 12 to 14, and then PLX 15 mg/kg on day 8 and 30 mg/kg on day 15 (Group 5).

Two perpendicular diameters (a and b) of the tumors were measured at regular intervals with a slide-square. Volume was calculated using the formula: $V = \pi ab^2/6$ [16].

Animals were sacrificed on day 23. Indian ink was injected through the trachea to stain the normal lung tissue, leaving the metastases as uncolored spots. Lungs were removed and immersed in glyofix fixative solution. The number of lung metastases was then counted under a binocular microscope [16].

Hematology

Blood counts were measured on the mouse on day 23 in a HMX Beckman coulter (Beckman Coulter, Fullerton, California, USA). Hematological parameters included neutrophils, blood platelets and lymphocytes. Blood formula percentages were evaluated on Giemsa-stained smears.

P-gp expression

P-gp expression was measured in the mouse lung by Western blot analysis. Lungs were removed 1 h after the last rIL-2 injection on day 7 (two mice from each group were sacrificed on day 7). Lung samples were homogenized using a glass Teflon potter in buffer (triethanolamine 10 mmol/l + 8.5% 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 bicinchoninic assay kit (Sigma-Aldrich, St-Quentin Fallavier, France), with BSA as a standard. Samples of 15 μg of proteins were separated by SDS–PAGE 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, Glostrup, Denmark) washed, and finally incubated with a peroxidase-conjugated anti-mouse IgG secondary antibody. The immunoreactive bands were visualized by the Enhanced Chemo-Luminescent system (Amersham, Little Chalfout, UK). The autoradiographs of P-gp protein were scanned and analyzed by densitometry using the Scion Image program to obtain a quantitative evaluation of the levels in the lung.

Statistical analysis

A Kruskal–Wallis ANOVA was carried out. If a significant difference was observed, a 2 × 2 statistical comparison test of the groups was performed (Bonferroni t-test).

Results

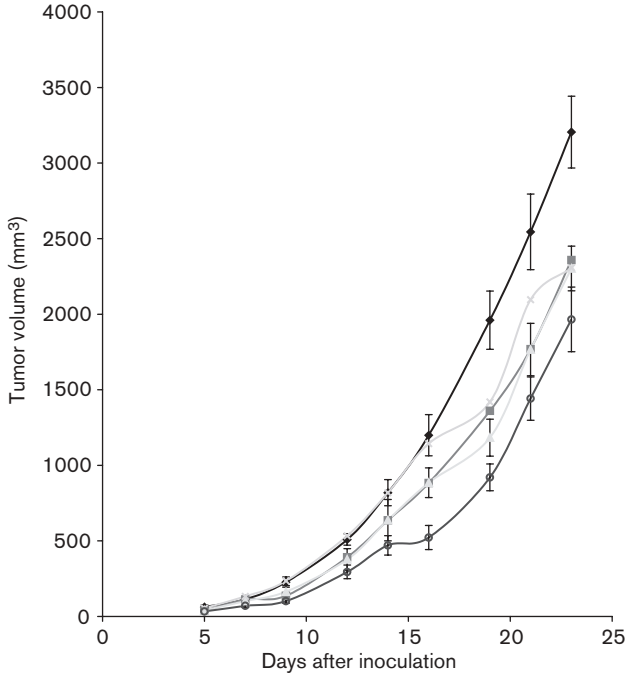
Influence of rIL-2 administration on paclitaxel efficacy on s.c. tumor growth

In mice who received rIL-2 twice daily from day 5 to 7 and from day 12 to 14, and then PLX 15 mg/kg on day 8 and 30 mg/kg on day 15 (Group 5), the average tumor volume was significantly lower than that of the control group (Group 1) on day 16 ($P < 0.01$) and on day 19 ($P < 0.01$, Bonferroni t-test) (Fig. 1). Other comparisons were not significantly different due to the very low bioavailability of PLX p.o.

In Group 3 (PLX alone) the tumor volume was significantly lower than that of the control group ($P < 0.05$) on day 19.

No significant difference was observed either between Group 3 (PLX alone) and Group 4 (rIL-2 i.p. twice a day on days 7 and 14, and then oral PLX on days 8 and 15) or between Groups 3 and 5 (rIL-2 i.p. twice a day from day

Fig. 1



Influence of rIL-2 administration on PLX efficacy on s.c. tumor growth. Group 1: control (diamonds). Group 2: rIL-2 from day 5 to 7 and from day 12 to 14 (squares). Group 3: PLX on days 8 and 15 (triangles). Group 4: rIL-2 on days 7 and 14, and then PLX on days 8 and 15 (crosses). Group 5: rIL-2 from day 5 to 7 and from day 12 to 14, and then PLX on days 8 and 15 (circles).

5 to 7 and from day 12 to 14, and then oral PLX on days 8 and 15) ($P > 0.05$).

RIL-2 i.p. twice daily from day 5 to 7 and from day 12 to 14, and then PLX 15 mg/kg on day 8 and 30 mg/kg on day 15 (Group 5) was the most efficient to control LLC s.c. growth, although it was not significantly better than PLX alone (Group 3).

Effects of the various treatments on the number of lung metastases

Treated mice were sacrificed on day 23, lungs were collected and stained with Indian ink, and metastases appearing as uncolored loci were counted under a binocular microscope. Data represent mean ± SD for each group.

All the rIL-2-injected groups had significantly lower numbers of lung metastases than those of the control (Group 1) and PLX-alone groups (Group 3) (Table 1).

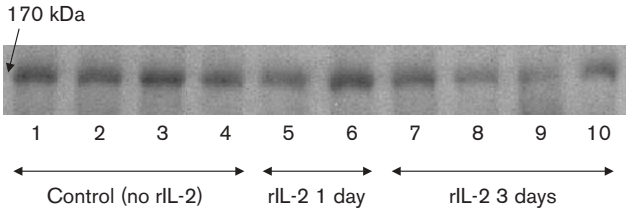
The smallest number of lung metastases was observed in Group 5, indicating that the combination rIL-2 for 3 days with PLX had the highest effect on metastases dissemination and development.

Table 1 Influence of rIL-2 administration on PLX efficacy on metastases development

Treatment	Lung metastases (mean ± SD)
Control	21.9 ± 3.6
rIL-2	6.9 ± 2.8[a]
Paclitaxel	13.5 ± 1.9[a]
rIL-2 1 day/paclitaxel	5.3 ± 1.5[a,b]
rIL-2 3 days/paclitaxel	1.9 ± 0.7[a,b]

[a]$P < 0.01$ versus controls (Bonferroni *t*-test).
[b]$P < 0.01$ versus paclitaxel alone (Bonferroni *t*-test).

Fig. 2



Immunodetection of P-gp from lungs of mice treated with rIL-2 for 1 or 3 days or no treatment. Lungs were collected 1 h after the last rIL-2 injection (on day 7) and lysed. Samples of 15 μg of protein were analyzed by Western blotting using the specific C219 anti-P-gp antibody. Control: from Group 1 (two mice) and Group 3 (two mice) (before PLX administration). rIL-2 1 day: from Group 4. rIL-2 3 days: from Group 2 (two mice) and Group 5 (two mice) (before PLX administration).

Table 2 Quantitative evaluation of lung P-gp expression

Treatment group	Quantitative evaluation of lung P-gp expression
Controls (no rIL-2)	6770 ± 309
rIL-2 1 day	6411 ± 515
rIL-2 3 days	4339 ± 480[a]

Autoradiographs of P-gp protein were scanned and analyzed by densitometry using the Scion Image program. Data represent means ± SD for each group.
[a]$P < 0.05$ versus controls.

Lung P-gp expression

The C219 anti-P-gp antibody we used recognized P-gp, the molecular mass of which is 170 kDa.

Western blot analysis of P-gp expression was performed in the lung of four control mice (two not treated from Group 1 and two from Group 3 before PLX administration) (Fig. 2, bands 1–4), two mice after 1 day of rIL-2 pre-treatment from Group 4 (Fig. 2, bands 5 and 6) and four mice after 3 days of rIL-2 (two from Group 2 and two from Group 5 before PLX administration) (Fig. 2, bands 7–10).

Immunoblots of P-gp protein were scanned to evaluate P-gp protein levels in the lung (Table 2). P-gp had decreased in lungs of mice which had received 3 days of the rIL-2 (Fig. 2, lanes 7–10). The immunoblot

Table 3 Hematological parameters in mice on day 23

Groups	Polynuclear neutrophils (G/L)	Platelets (× 103 g/l)	Lymphocytes (G/L)
Control (Group 1)	4803 ± 440	631 ± 34	9680 ± 930
rIL-2 (Group 2)	5730 ± 1620	657 ± 63	8960 ± 900
Paclitaxel (Group 3)	5449 ± 720	764 ± 87	8870 ± 820
Paclitaxel/rIL-2 1 day (Group 4)	9564 ± 2870	807 ± 75	10616 ± 1370
Paclitaxel/rIL-2 3 days (Group 5)	4650 ± 610	657 ± 104	8480 ± 615

Blood was collected on day 23 from each mouse and analyzed on a Beckman coulter counter. Data represent means ± SD for each group.

densitometry value of group treated with rIL-2 for 3 days was statistically lower than that of the control group ($P < 0.05$), but not significantly different from that of the group treated with rIL-2 for 1 day.

Hematological toxicity

No difference in hematological toxicity (polynuclear neutrophils, platelets and lymphocytes) was observed between the five groups on day 23 (Table 3). PLX treatment alone or in association with rIL-2 had no side-effects on mouse blood formulation as there was no decline in blood cell counts under therapy.

Discussion

Preclinical studies with the murine Madison 109 lung carcinoma (M109) model indicate that optimum i.v doses of PLX per injection ranged from 24 to 36 mg/kg [17]. We gave a lower dose p.o. to allow a pre-treatment to increase PLX anti-tumor activity. The highest anti-tumor activity was observed after a pre-treatment with rIL-2 for 3 days followed by doses of PLX (15 and 30 mg/kg) administered p.o. without any increase of hematological toxicity.

Mice received rIL-2 for 3 days because preliminary experiments had shown that 1 day of pre-treatment was not enough to inhibit intestinal P-gp.

The group treated with PLX + rIL-2 for 3 days developed the lowest number of lung metastases and the least voluminous s.c. tumors. The group that received rIL-2 alone had a significantly smaller number of lung metastases than the control group, indicating that rIL-2 in itself has anti-cancer activity [13]. Consequently, it is reasonable to think that this increase in anti-cancer activity is due to the addition of the anti-cancer effect of PLX and rIL-2 iterative doses.

In mice treated for 3 days with rIL-2, lung P-gp expression measured 1 h after the last rIL-2 injection was significantly decreased, thus explaining the better anti-tumor activity of the association and in agreement with a recent study in 40 patients, indicating that the response to treatment by PLX of bronchial cancer patients is closely related to the level of expression of lung P-gp [9,18].

Other effects of rIL-2 could be discussed such as an increase of vascular permeability (capillary leaking syndrome) [19] allowing a better penetration of PLX in the tissues, particularly in tumors. This side-effect of rIL-2 was not investigated in this study. By facilitating absorption and distribution of PLX, rIL-2 could modify the effect of PLX in three ways; (i) by increasing the bioavailability of oral PLX by inhibiting intestinal P-gp as demonstrated previously [15], (ii) by reducing the efflux effect of P-gp in tumor cells and/or (iii) by increasing vascular permeability.

Cytokine-associated chemotherapy could have an interesting effect when compared to immunotherapy on resistant metastasis areas (liver, brain).

In addition, the association of the two treatments did not increase hematological toxicity, whereas other P-gp inhibitors do, such as cyclosporine, which increased the bioavailability of substrates of P-gp [7].

It would be interesting to determine the feasibility of associating rIL-2 and PLX in clinical studies.

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