Effect of PSC 833, an inhibitor of P-glycoprotein on N-methyl-N-nitrosourea induced mammary carcinogenesis in rats

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Studies in our laboratory on the role of P-glycoprotein (Pgp, coded by mdr1 gene) have led to the hypothesis that over-expression of Pgp is closely associated with the development of cancer. It was conceived therefore that inhibitors of Pgp should inhibit the development of cancer. We have reported that PSC833 (PSC), a potent inhibitor of Pgp, inhibits the development of liver cancer in rats. Similarly, based on the intrinsic over-expression of Pgp in experimental mammary carcinogenesis, we studied the effect of PSC on N-methyl-N-nitrosourea induced mammary cancer in female Sprague–Dawley rats. The study indicates that PSC at daily dietary doses of 15 (PSC15) and 30 mg/kg (PSC30) body wt resulted in dose-dependent inhibition of the incidence as well as the growth of mammary tumors. Compared with controls, PSC15 and PSC30 inhibited: (i) mean tumor multiplicity by 32 and 67%, (ii) median tumor burden by 46 and 93% and (iii) incidence of ulcerated tumors by 40 and 82%, respectively. Most remarkably, PSC delayed median tumor incidence by 8 weeks, and exerted a 100% inhibitory effect on the incidence of large tumors, 4 cm³ and greater. In all the cases, although the inhibitory effect of PSC was evident at both doses, only PSC30 exhibited statistical significance. A possible compounding effect that was also observed in PSC30-treated rats was a decrease in body weight gain not attributed to diminished food consumption. All in all, consistent with recent reports, which have demonstrated inhibition of cancer development by compromising Pgp function, this study introduces a novel role for Pgp in breast cancer and potentially an unexplored therapeutic approach in treating the disease.

Materials and methods

Reagents

PSC was a gift from Novartis. MNU (Sigma Chemical Co., St Louis, MO) was freshly dissolved in 0.9% sodium chloride adjusted to pH 6.7 with 0.05% acetic acid to give a concentration of 10 mg MNU/ml, and administered within 2 h of preparation.

Animal care

The experimental protocol used was approved by the University of Toronto Animal Care and Use Committee. Rats were housed individually in solid bottom cages with corncob bedding, at 22 °C and 50% humidity with 12 h light–dark cycle. The rats had free access to water and diet.

Effect of PSC on MNU-induced mammary tumors in rats

Forty-day-old specific pathogen-free Sprague–Dawley female rats (Charles River, St Constant, Quebec, Canada) were fed Purina rat chow diet upon arrival and were later switched to basal diet AIN-93G (AIN, #110700, Dyets, Bethlehem, PA). At day 50, all rats received a single dose of MNU (50 mg/kg i.p.). Two weeks post-MNU administration, a time by which the animals would have recovered from the MNU-induced toxicity, the rats were divided into three groups. Rats in Group 1 were exposed to basal diet AIN (n = 21), while rats in Groups 2 and 3 were exposed to AIN diet containing two different concentrations of PSC. PSC was pre-mixed at 15 (PSC15, n = 20) and 30 mg/kg body wt (PSC30; n = 17), respectively, based on an average diet consumption of 14.0 g/day/rat. Body weight and diet consumption of the rats were monitored every week. Beginning 6 weeks post-MNU administration, the rats were palpated every week for the evidence of tumors. The position of palpable tumors was categorized as left or right cervical, thoracic, abdominal or inguinal. Dimensions (length (L) × width (W) × depth (D)) of the tumors were measured with a pair of digital calipers, and the volume was calculated using the following formula: 0.5238 × L × W × D. Rats that developed ulcerating tumors before the termination of the experiment were killed within a day after diagnosis. At the termination of the experiment, 26 weeks post-MNU, the rats were killed by carbon dioxide asphyxiation and a detailed necropsy was performed on each reported in many untreated human cancers such as in breast, liver, colon, kidney and brain (2–8). Taken together, these observations suggest that increased expression of Pgp in cancers plays a role in cancer development. It is probable therefore, that inhibition of Pgp function could inhibit the development of cancer. In an earlier study on liver carcinogenesis we showed that PSC833 (PSC, Novartis), a potent inhibitor of Pgp inhibited the development of liver cancer induced by 1,2-dimethylhydrazine in the rat (9). Human breast cancers often over-express Pgp (2,4,5,7,10–13). Several studies have correlated Pgp expression in breast carcinoma with poor prognosis (11,12). In human breast carcinomas, Pgp expression in neoplastic epithelial cells appears to be a marker of a more malignant phenotype especially when accompanied by stromal expression (13). Furthermore, experimental mammary tumors induced in rats by N-methyl-N-nitrosourea (MNU) also over-express Pgp (8) and the increased expression of Pgp is seen particularly at the epithelial–stroma interface. These results suggested that Pgp expression might confer an invasive advantage. Based on these considerations, the present study was undertaken to determine whether PSC, an inhibitor of Pgp would inhibit MNU-induced mammary cancer development in the rat.

Introduction

Our study on the role of P-glycoprotein (Pgp, coded by mdr1 gene) in the carcinogenic process has led to the hypothesis that its over-expression is intimately associated with the development of cancer. This hypothesis is based on our findings that over-expression of Pgp begins early in experimental liver carcinogenesis and increases with the progression of the disease (1). High expression of the mdr1 gene has also been

Abbreviations: MNU, N-methyl-N-nitrosourea; Pgp, P-glycoprotein; PSC, PSC833.
rat. Blood was drawn from the common femoral artery at the bifurcation, and the serum was stored at −80°C. Location, weight and dimensions of excised mammary mammary tumors were recorded. Tumors larger than 1 cm³ in volume were sliced along the middle and fixed in 10% phosphate-buffered formalin, embedded in paraffin blocks and processed for histological examination of adenomas and adenocarcinomas using criteria described by Russo et al. (14). Briefly, histopathologic criteria used to determine malignancy were: (i) loss of tubular-alveolar pattern of the normal mammary gland, (ii) presence of large epithelial cells with increased nucleo-cytoplasmic ratio, (iii) stromal response by fibrosis and inflammatory cell infiltration and (iv) necrosis and hemorrhage. In each case, the remaining tumor tissue was instantly frozen in liquid nitrogen and stored at −80°C for further analysis. The kidneys, liver, lungs and surrounding mammary tissues were also removed and stored for histological and biochemical analyses.

**Western blot analysis of Pgp**

Frozen mammary tumor tissues (0.5 g) were thawed to room temperature, homogenized in 3 ml HEPES–mannitol buffer containing 50 mM mannitol, 20 mM HEPES, 10 mM β-mercaptoethanol, 1:1000 dilution of protease inhibitor cocktail (P-8340, Sigma Aldrich, St Louis, MO) and centrifuged at 15 000 g for 15 min. The supernatant was then centrifuged at 100 000 g for 60 min. The microsomal pellet was resuspended and dissolved in the HEPES–mannitol buffer. Total protein content in tissue lysates was estimated using the Bio-Rad protein assay kit with bovine serum albumin as the standard. The microsomal fractions (100 and 200 μg of protein) were electrophoresed in 8% SDS–PAGE according to standard procedures. The samples were then blotted to nitrocellulose paper and immunoprobed using C219 (1:3000, Fujirebio Diagnostics, Malvern, PA), a monoclonal antibody, which recognizes the different isoforms of Pgp. The blots were developed using the ECL Kit (Amersham Pharmaceuticals, Piscataway, NJ). Pgp expression was represented by a ~170 kDa band on the SDS–PAGE.

**Statistical analysis**

Incidence of tumors (expressed as the percentage of tumor bearing rats per group) or ulcerated tumors (expressed as the percentage of rats killed for ulcerated tumors per group) were compared among the groups using the Fisher’s exact contingency test. Cumulative tumor incidence was assessed in a time-dependent manner using Kaplan–Meier Life Table Curves. To generate these curves, rats that produced the first detectable tumor were assigned a score of 1, while those that failed to develop tumors until the end of study were given a score of 0. Three rats in the PSC30 group were killed for PSC-induced morbidity and were excluded from all analyses performed in the study. The scores were then tabulated based on the time of tumor detection, represented as weeks post-MNU administration. The curves of the treatment groups were then compared with those of the control group using the Log Rank test. All other pair-wise comparisons were statistically non-significant. Note that 12 weeks post-MNU treatment, the rate of gain in body weight in PSC30 group is comparable with that in the control AIN group. (13,15). These discrepancies were attributed to the different methods of Pgp detection and also to variations in the definitions of positivity within a given method of detection (16,17).

A recent meta-analysis performed on 31 studies to assess functional significance of Pgp expression in breast cancers indicated that 41.2% of breast cancers express Pgp/mdr1 (18). The results of the present study indicate that the MNU-induced mammary tumors exhibited increased expression of Pgp compared with their corresponding non-lesion surrounding mammary tissue. We have screened over 15 mammary tumors in the present study and all of them exhibited increased Pgp expression. These results are in agreement with those published earlier (8). Western analysis of Pgp expression in tumors compared with surrounding from four representative rats is presented in Figure 3. Interestingly, the surrounding tissue exhibited a C219 cross-reacting band of a slightly higher molecular weight than Pgp. At present we have not characterized this protein or its relationship to the 170 kDa Pgp band found in the tumor tissue.

To determine whether PSC exerted any inhibitory effect on mammary tumor development, cumulative tumor incidence was plotted using a Kaplan–Meier Life Table Curve. The results presented in Figure 4 show that in the PSC30 group, rats had significantly decreased incidence of tumors and the inhibition was persistent until the end of the study. Further, the median tumor incidence was extended from 16 weeks in the controls to 24 weeks in PSC30. While PSC15 did not exhibit an inhibitory effect on the incidence of mammary tumors when expressed as number of tumor bearing animals (Figure 4), it clearly had an effect on tumor multiplicity (Figure 5). Compared with controls, PSC15 and PSC30 inhibited the number of tumors per rat by 31.6% and 66.7%, respectively. To determine if the treatment inhibited the growth of the tumors as well, tumor burden, expressed as total tumor volume per rat, was assessed between different groups. As presented in
Figure 6. PSC30 significantly inhibited the total volume of tumors per rat by 81.6% compared with controls, while PSC15 exhibited a marginal inhibitory effect. This marginal effect of PSC15 could be due to two rats, which showed a very high tumor burden, uncharacteristic of the rest of the rats in this group. Because of this we calculated the median, which is more representative of this group. The median tumor burden for the AIN control, PSC15 and PSC30 were 3.90, 2.11 and 0.28, respectively. Thus, the median values showed that PSC15 decreased tumor burden by 46% and PSC30 by 93%. Results presented in Figure 7 show the inhibitory effect of PSC on the distribution of tumor number categorized by their volume in the three groups. Most remarkably, PSC30 completely inhibited the incidence of large tumors (4 cm$^3$ and greater). Furthermore, rats that developed severely ulcerated mammary tumors during the course of the study were killed within a day of diagnosis. The number of rats killed for ulcerated mammary tumors in each group is shown in Figure 8. Compared with controls where seven rats (33.3%) were prematurely killed for severely ulcerated mammary tumors, there...
were only four rats (20%) in PSC15 and one rat (5.9%) in PSC30. Consistent with these observations, microscopic analysis indicated that compared with controls where 73% of the tumors were adenocarcinomas, only 54% in the PSC15 group and 24% in the PSC30 group were adenocarcinomas (Figure 9). The tumors are ductal carcinomas with varying combinations of papillary, cribriform and comedo patterns. The site-distribution of tumors in PSC30-treated rats was similar to that of controls, thereby suggesting that PSC30-induced tumor inhibition may not be site-specific. In summary, although tumor inhibition by PSC was dose-dependent, statistically significance was achieved only in the PSC30 group.

Discussion

The over-expression of Pgp in cancer development appears to be both an intrinsic and an acquired phenomenon. Acquired expression of Pgp often follows exposure to drugs and is thought to play a role primarily in drug-efflux. As such, Pgp modulators currently used in experimental research as well as in clinical trials, act as adjuvant agents for other chemotherapeutic drugs (19–22). However, the intrinsic expression of Pgp in tumors appears to play a more fundamental role in promoting tumor growth and development. Therefore, tumors that intrinsically over-express Pgp during development, such as tumors in MNU-induced mammary carcinogenesis in rats, should conceivably be sensitive to Pgp inhibition. The results of the present study are in support of this consideration in that PSC, a potent inhibitor of Pgp, given at daily dietary doses of 15 and 30 mg/kg body wt inhibited the incidence as well as the...
Inhibition of mammary carcinogenesis by PSC 833

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References


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