PSC 833, an Inhibitor of P-glycoprotein Inhibits 1,2-Dimethylhydrazine-induced Colorectal Carcinogenesis in Male Fischer F344 Rats

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Abstract. *Background: The expression of P-glycoprotein (Pgp) is intimately associated with cancer development. In order to explore the therapeutic value of Pgp as a target for chemotherapy, we studied the effect of PSC 833 (PSC), a potent inhibitor of Pgp, on 1,2-dimethylhydrazine (1,2-DMH)-initiated colorectal carcinogenesis in rats. Materials and Methods: Male Fischer 344 rats, initiated with 1,2-DMH coupled with partial hepatectomy, were exposed to dietary 1% orotic acid for 22 weeks. They were then fed either the AIN93G basal diet (BD) or BD containing PSC (a daily dose of 15 mg/kg body weight) for 35 weeks. Results: PSC significantly inhibited colorectal tumor multiplicity by 53% and tumor burden by 74%. PSC-mediated inhibition was evident in tumors as small as 2 mm in diameter and remained effective throughout the course of tumor growth. Histological assessment showed that PSC significantly inhibited tumor progression to colorectal adenocarcinoma by 63%. Conclusion: Collectively, this study indicates that PSC inhibited experimental colorectal carcinogenesis initiated with 1,2-DMH in rats.*

P-glycoprotein (Pgp, product of mdr1, also designated as ABCB1) is a member of a super-family of ATP-dependent membrane transporters (1, 2). We have been working on the hypothesis that, in addition to conferring multi-drug resistance to a wide variety of late stage cancers, overexpression of Pgp is associated with the development of cancers. This hypothesis stems from the observations that high expression of the *mdr1* gene has been reported in a significant percentage of a wide variety of untreated human cancers including colorectal tumors. For example, 30-96% of human colorectal cancers at the time of diagnosis overexpress Pgp (3-5). Weinstein *et al.* (5) observed a statistically significant association between Pgp expression in a specific sub-population of colon cancer cells and a high prevalence of vessel invasion and lymph node metastases. It should be pointed out, however, that there are a few reports of human cancers in which increased expression of Pgp/mdr1 was not seen. At least in some of these instances, the discrepancies were attributed to the different methods of Pgp detection and variations in the definitions of positivity within a given method of detection (6). In addition to the findings from human cancers, several experimental carcinogenesis studies also indicated that overexpression of Pgp starts early in the carcinogenic process, and its expression increases with progression of the disease (7-9). For example, our earlier studies on experimental rat liver carcinogenesis indicated that Pgp was overexpressed in early hepatic nodules induced by 1,2-dimethylhydrazine (1,2-DMH) and the number of nodule hepatocytes overexpressing Pgp increased with the progression of the disease (7). Recently, it has been shown that imposing mdr1ab$^{-/-}$ on *min* genotype (A*pc*$^{min/+}$mdr1ab$^{-/-}$) resulted in significantly fewer intestinal polyps compared to the A*pc*$^{min/+}$mdr 1a/b$^{+/+}$ mice (10, 11). These observations strongly indicate that overexpression of Pgp is intimately associated with cancer development. If overexpression of Pgp is important for cancer development, then, inhibitors of Pgp should inhibit cancer development. Consistent with this hypothesis, we have previously demonstrated that PSC 833 (PSC, also known as Valspodar, from Novartis), a potent inhibitor of Pgp, inhibited the development of 1,2-DMH-induced liver and *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in rats (12, 13). In this communication, we present results to show that PSC inhibits yet another organ specific cancer – colorectal carcinogenesis induced by 1,2-DMH in rats.

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Materials and Methods

Male Fischer 344 rats (170-180g) were individually housed in solid bottomed cages with corncob bedding, at 22˚C and 50% humidity, with 12-hour light-dark cycle. The rats had free access to water and diet. After one week in our facility, the rats were exposed to AIN-93G basal diet (BD, Dyets Inc., Bethlehem, PA, USA) and were initiated with a single dose of 1,2-DMH (Sigma Chemical Co., St. Louis, MO, USA). 1,2-DMH.2HCl was freshly dissolved in 0.9% sodium chloride containing 0.015% EDTA, adjusted to pH 7.0 and given at a dose of 100 mg/kg; *i.p.* 18-20 hours after two-thirds partial hepatectomy (PH). Two-thirds PH was performed under anesthesia with Isoflurane (5% for induction and 2% for maintenance by inhalation) gas in oxygen saturated medium. As a post-surgical analgesic, Buprenorphine was given subcutaneously at a dose of 0.04 mg/kg body weight. After 2 weeks of recovery, the rats were exposed for 22 weeks to a tumor-promoting diet (BD plus 1% orotic acid) (14). At this point, the rats were transferred to BD to clear the circulating orotic acid. Two weeks thereafter, the rats were divided into 2 groups. One group continued on the BD (n=24) and the other group was transferred to BD containing PSC (gift from Novartis) to deliver a daily dose of 15 mg/kg body weight (PSC; n=18). The rats were euthanized by carbon dioxide asphyxiation after 35 weeks on the PSC diets. At necropsy, the colons were removed, slit open, washed free of fecal material and the size and location of colonic tumors were recorded. The liver and lungs from all the rats were also processed for gross and histological assessment. Lesions present on the surface as well as on the cut surface were counted and their size measured. Colorectal lesions were classified as adenomatous polyps or colorectal adenocarcinomas (CAC), based on the established criteria (15).

Results

The mean body weight of the rats and diet consumption were comparable in both groups, indicating that PSC did not exert any significant toxic effects. Mean body weights at necropsy in the BD and PSC groups were 372±27 and 388±33 g (±SD), while the mean daily diet consumption was 16.2±0.7 and 15.3±1.1 g (±SD), respectively. Quantitative data of the rat colorectal tumors extracted from the control BD and PSC treatment groups are represented in Figure 1 with respect to the size and location of the tumors. The majority (over 85%) of the colorectal lesions were located in the distal region of the colon, a finding consistent with literature reports (16, 17). More importantly, the figure indicates that PSC inhibited the development of colorectal tumors. The median tumor size in the BD group was 6.5 mm. Compared to 50% in the BD group, only 19% of the lesions in the PSC treatment group were larger than 6.5 mm, suggesting that the inhibition exerted by PSC was significant. To better assess the PSC-mediated inhibition of colorectal tumor development, the data were represented as tumor multiplicity and burden, as shown in Figure 2. These results demonstrated that compared to the control, PSC inhibited colorectal multiplicity by 53% and tumor burden by 74%. As displayed, this inhibition was statistically significant by unpaired two-tailed *t*-test analyses. To further characterize the effect of PSC on the growth of these lesions, the distribution of lesions in a size-specific manner was determined, as illustrated in Table I. The results presented in Table I indicate that the inhibitory effect of PSC was evident in tumors as small as 2 mm in diameter, suggesting an early inhibition. Interestingly, the table also demonstrates that in the PSC treatment group, the percent incidence of progressively larger lesions (2, 4, 6 and 8 mm) tapered to zero at 10 mm in diameter. This indicates that the inhibition of colorectal tumors by PSC not only starts early, but remains effective for prolonged periods of time during the course of tumor development. This conclusion was further strengthened when histological assessment of the tumors showed that the incidence of colorectal adenocarcinomas (CAC) was significantly reduced in the PSC treatment group compared to the controls. The incidences of CAC in the BD and PSC groups were 75% (18/24) and 28% (5/18), respectively, indicating that PSC inhibited the progression of lesions to CAC by a significant 63%. The collective analyses of results presented in this study suggest that PSC inhibited the growth and progression of colorectal carcinogenesis in 1,2-DMH-initiated rats.

Table I. *Effect of PSC on percent distribution of size-specific colorectal tumors.*

Tumor diameter (size)	BD	PSC
2 mm and smaller	15	43$^{\delta}$
Larger than 2 mm	85	57$^{\delta}$
Larger than 4 mm	58	33
Larger than 6 mm	50	19$^{\delta}$
Larger than 8 mm	30	5$^{\delta}$
Larger than 10 mm	13	0

To assess the inhibitory effect of PSC on the growth of colorectal tumors, several arbitrary size categories (every 2 mm) were made using the results presented in Figure 1. The statistical significance of the effect of PSC was calculated by comparing the raw tumor numbers in each size category from PSC-treated groups with those of BD, by using Fisher's exact contingency test. $^{\delta}p<0.05$, compared to BD controls.

Discussion

In the present study, PSC was given 22 weeks after the administration of 1,2-DMH. The inhibitory effect of PSC could, therefore, mean that PSC inhibited the growth of pre-existing lesions. This is reflected by the inhibitory effect of PSC on the growth and progression of the colonic lesions. These results are in agreement with our earlier observations that PSC also inhibited liver and mammary carcinogenesis induced by 1,2-DMH and by *N*-methyl-*N*-nitrosourea, respectively (12, 13). Recently, PSC has also been shown to

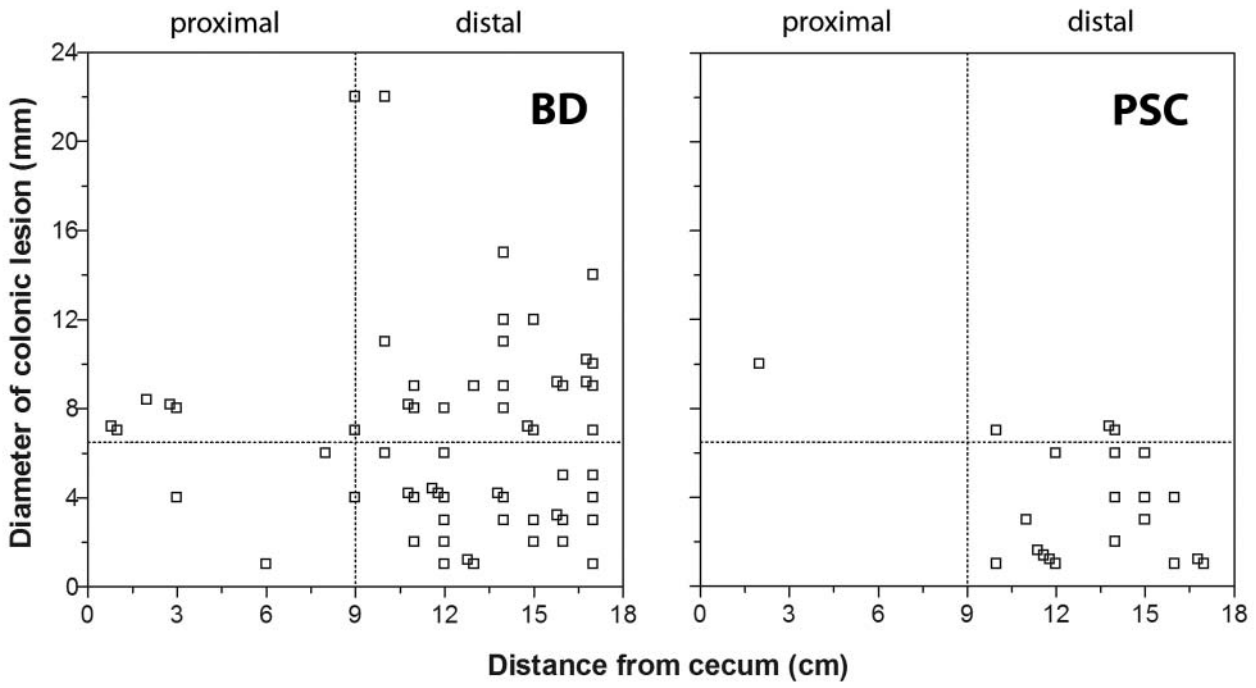

Figure 1. *Effect of PSC on the size and location of colorectal tumors. Each square dot represents a tumor with respect to its size (largest diameter in mm) and location (distance from the cecum in centimeters). The colon length was arbitrarily divided at 9 cm (~ mid point) to categorize tumor location as proximal (at the cecum end) or distal. The horizontal dotted line at 6.5 mm represents the median tumor diameter in the BD group. All grossly visible colorectal tumors were counted, measured and are represented in the figure.*

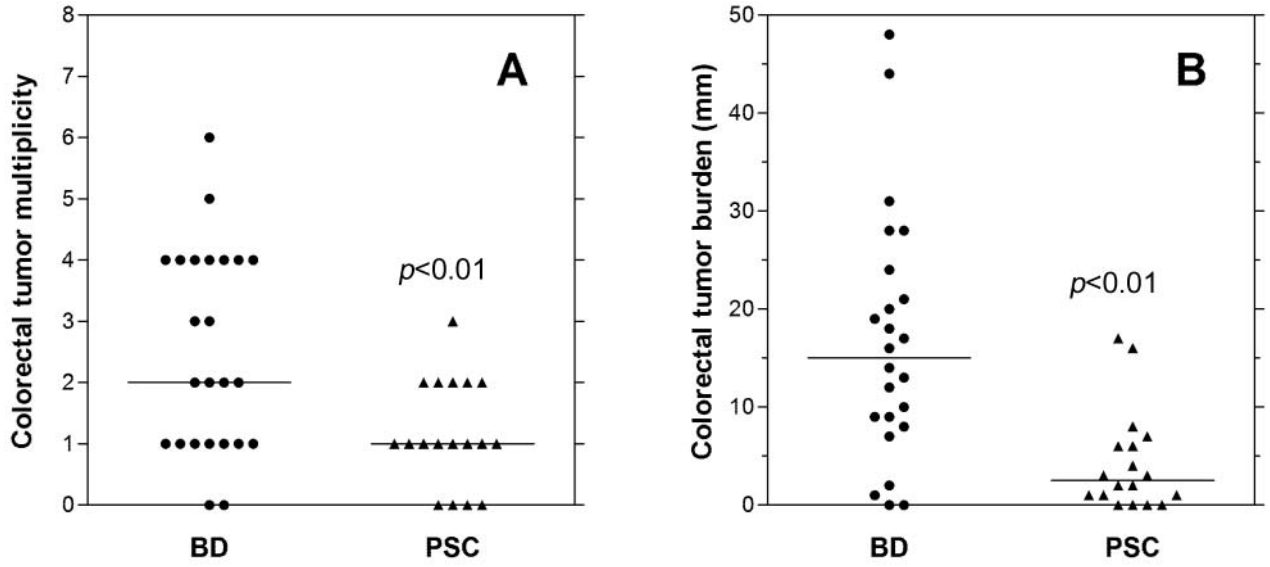

Figure 2. *Effect of PSC exposure on colorectal tumor (A) multiplicity and (B) burden in rats. Each dot represents a rat. Plot (A) shows the total number of tumors per rat, while (B) depicts the total tallied diameter of lesions per rat. The horizontal bar displays the median for each group. $p<0.01$ denotes statistical significance by unpaired two-tailed t-test analyses. The results indicate that PSC significantly inhibited colorectal tumor multiplicity by 53% and tumor burden by 74%.*

inhibit the engraftment of KG1a/200 human leukemia cells in immune deficient mice (18). In addition, PSC has been shown to induce apoptosis and/or cell cycle arrest in several tumor cell lines (19-21). Taken together, these results clearly suggest that PSC has the potential to be an excellent multi-organ tumor inhibitor.

From a mechanistic point of view, PSC can exert its tumor inhibitory effect by way of its ability to inhibit Pgp or by a mechanism independent of Pgp expression. Recently, it was reported that mdr1-deficient *min* ($Apc^{min/+}$mdr1ab$^{-/-}$) mice developed significantly fewer intestinal polyps than $Apc^{min/+}$mdr 1ab$^{+/+}$ mice (10, 11). These results lend further support to the concept that Pgp plays an important role in intestinal carcinogenesis and PSC might exert its tumor inhibitory effecτ by virtue of its ability to inhibit Pgp. In addition to being an efflux pump, Pgp also plays important roles in cell cycle, differentiation and apoptosis (1, 22, 23). For example, several lines of evidence indicate that Pgp confers protection from ceramide-mediated apoptosis (19, 22, 24). In addition, it has also been shown that PSC-induced apoptosis and/or cell cycle arrest is associated with increased levels of ceramide (19, 20), although the causal relationship between PSC-induced increased levels of ceramide and PSC-induced apoptosis and/or cell cycle arrest has not yet been established. Furthermore, ceramide and its metabolites have been shown to inhibit colorectal and liver carcinogenesis (25-27). Pgp may thus support tumor development by influencing one or more of these functions and PSC, by virtue of its ability to inhibit Pgp, can inhibit tumor development. However, more work is needed to implicate any particular mechanism by which PSC exerts its novel multi-organ tumor inhibitory effect.

A serendipitous finding of interest is that, in the present study, initiated rats developed both colorectal and liver cancers. Conventionally 1,2-DMH is considered to be a potent colon carcinogen (16, 17). However, when its administration is coupled with two-thirds PH, 1,2-DMH becomes an excellent liver carcinogen (14, 28). Previous studies from our laboratory have shown that rats initiated with 1,2-DMH coupled with two-thirds PH developed at the most only a 15% incidence of colon tumors (14, 28). Surprisingly, in the present experiment, 90% of rats receiving 1,2-DMH developed colorectal lesions at the end of 56 weeks, while the incidence of liver tumors, as in the earlier studies, remained over 90%. A reason for this serendipitous finding may be attributed to two changes in the original protocol. In our earlier studies (14, 28), ether (inhalation) was used to anesthetize rats for two-thirds PH. In the present study, however, Isoflurane was used as the anesthetic. In addition, Buprenorphine was given as the post-surgery analgesic. We believe that the change in anesthetic and/or the analgesic used in the present experiment might have contributed to this dramatic shift in carcinogen sensitivity.

In conclusion, our studies that PSC can inhibit carcinogenesis in the liver (13), mammary gland (12) and colon (the present study) strongly suggest that PSC and possibly other inhibitors of Pgp by themselves may be used as cancer chemopreventive agents.

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