Medicinal Chemistry of Probimane and MST-16: Comparison of Anticancer Effects Between Bisdioxopiperazines

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Abstract: Bisdioxopiperazines, including ICRF-154 and razoxane (ICRF-159, Raz), are a family of anticancer agents developed in the UK, specifically targeting neoplastic metastases. Two other bisdioxopiperazine derivatives, probimane (Pro) and MST-16, were synthesized at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. In order to determine the similarities and differences between these agents in medical chemistry, we evaluated the anti-tumor and anti-metastatic effects of Pro and MST-16 *in vitro* and *in vivo* against a number of human tumor cell lines and one of murine origin (Lewis lung carcinoma, LLC), and one human tumor xenograft (LAX-83) in nude mice.

Our results show that Pro was cytotoxic to human tumor cell lines *in vitro* (IC_{50} < 50 μM for 48 h), approximately 3 to 20-fold more than MST-16. Pro and MST-16 manifested more prolonged cytotoxicity than some other first-line anticancer drugs including 5-fluorouacil, vincristine and doxorubicin, and maintain their cytotoxic effects for 4 days *in vitro*. In animal experiments, Pro and Raz were active against primary tumor growth (35-50 %) and significantly inhibited pulmonary metastasis of LLC (inhibition > 90 %) at dosage below LD_5. Both Raz and Pro were effective in administration schedules of 1, 5 and 9 days. Both Raz (25-32 %) and Pro (55-60 %) caused statistically significant inhibition of the growth of LAX 83 (a human lung adeno-carcinoma xenograft) in nude mice. In this model, Pro was more effective against LAX83 than Raz at equitoxic dosages.

These findings suggest that Pro is active against more categories of tumors both *in vivo* and *in vitro*, which in some circumstances may make it superior to the currently-used anticancer bisdioxopiperazines, including razoxane and MST-16.

INTRODUCTION

Bisdioxopiperazines, including ICRF-154, razoxane (ICRF-159, *Raz*), ICRF-186 and ICRF-187 (two stereoisomers of Raz) and ICRF-193, developed in the UK, were among the earliest agents found to be effective against a model of spontaneous metastatis (Lewis lung carcinoma) [1]. Since that time (1969), many studies have addressed their potential use and mechanisms of action. Three main mechanisms of action have been investigated: potentiating the effect of radiotherapy [2-3], overcoming multi-drug resistance (MDR) to daunorubicin and doxorubicin in leukemia [4-5], and inhibiting topoisomerase II [6-7]. More importantly, *Raz* has been licensed in many countries as a cardioprotectant during anthrocycline treatment. Since bisdioxopiperazines are unique agents in that their pharmacological action is conser-vative, probimane [1,2-bis (N^4-morpholine-3, 5-dioxopeprazine-1-yl)propane; AT-2153, Pro] and MST-16 [1,2-bis(4-isobutoxy-carbonyloxymethyl-3,5-dioxopiperazin-1-yl)ethane] were synthesized at this institute in Shanghai, China [8-9]. The structural formulae of the three bisdioxopiperazines are represented in Fig. **1**. In addition to data on anti-tumor activity [10-12], Pro has been shown to have similar pharmacological mechanisms and actions to Raz, such as amelioration of *Adriamycin* (*ADR*)-induced cardiotoxicity and synergism with *ADR* against leukemias, as reported from the Henan Academy of Medicine, Henan, China [13]. As the principal researchers on Pro, we have reported some novel biological effects of this compound including inhibition of the cell signal regulator calmodulin (*CaM*), which might explain its anticancer activity; its cytotoxic effect in combination with *ADR* [14]; its inhibition of erythrocyte lipoperoxidation (*LPO*) [15]; its down-regulation of tumor sialic acid synthesis [16]; and its blocking of fibrinogen binding to leukemia cells [17]. MST-16, as a licensed drug in Japan since 1994, is allowed for direct use in leukemia chemotherapy, mainly against adult T-cell leukemia [18-19].

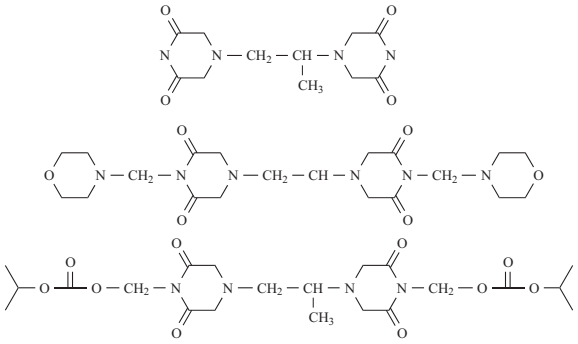

Fig. (1). Structural formulae of bisdioxopiperazines.

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As a new bisdioxopiperazine, the pharmacological characteristics of Pro are intriguing. Increased understanding and comparison of the two compounds are the first steps towards promoting applications of Pro and MST-16. This article gives details of the anti-proliferation and cytotoxic data obtained in this laboratory, including in-depth pharmacological evaluation and IC_{50} values for 10 human tumor cell lines from 7 different organs of origin: two leukemia lines (HL-60 and K562), two gastric tumor lines (SCG-7901 and MKN-28), a lung tumor line (A549), a colon cancer line (HCT-116), two mammary tumor lines (MDA-MB-435 and MDA-MB-468), a hepatic tumor cell line (BEL7401) and an uteric cervical tumor cell line (HeLa). The anticancer activities and anti-metastatic efficacies of Pro and Raz *in vivo* were also compared.

RESULTS

1. Anti-Proliferative Effects of Pro and Other Bisdioxopiperazines *In Vitro*

We determined the IC_{50} values of probimane against 10 human tumor cell lines originating from 7 organ types (Fig. **2**) using the MTT assay, and we compared the IC_{50} values of MST-16 and probimane for several of these tumor types

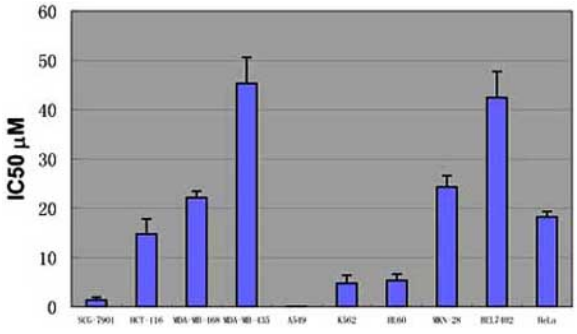

Fig. (2). Anticancer activities of probimane *in vitro*; MTT method. Cells were exposed to Pro for 48 hours at 5 different concentrations; n = 3 in two independent tests.

(Fig. **3**). These data show that probimane had greater cytotoxic effects than Raz or MST-16, especially against solid tumors (Figs. **4**-**6**). Also, the cytotoxic effects of probimane and MST-16 steadily increased over 72 h. Comparisons of cytotoxic effects between bisdioxopiperazines and other anticancer drugs are shown in Tables **1**-**4**. These data show that Pro was effective at lower doses and in shorter times.

2. Anticancer and Anti-Metastatic Effects of Raz and Pro Against a Murine Tumor Model (Lewis Lung Carcinoma) *In Vivo*

The antitumor and antimetastatic effects of Pro and Raz on LLC are shown in Tables **5** and **6**. Pro and Raz at equitoxic doses (LD_5) showed a noticeable anticancer effect on primary tumor growth (inhibition approximately 30-45 %), and significantly inhibited the formation of tumor metastases (inhibition of pulmonary metastasis >90 %, $P<0.001$). Primary LLC tumor growth was inhibited more by Pro (48 %) than by Raz (40.3%) in a 20 day trial, whereas

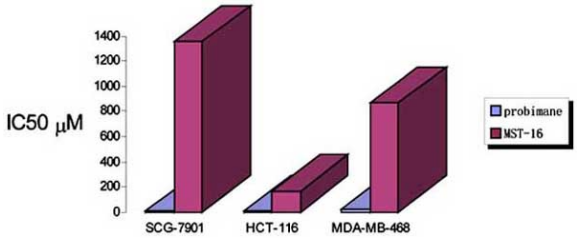

Fig. (3). The IC_{50} values of probimane and MST-16 tested by exposure of three human gastrointestinal tumor cell lines (SCG-7901, HCT-116 and MDA-MB-468) for 48 h. MTT method was used.

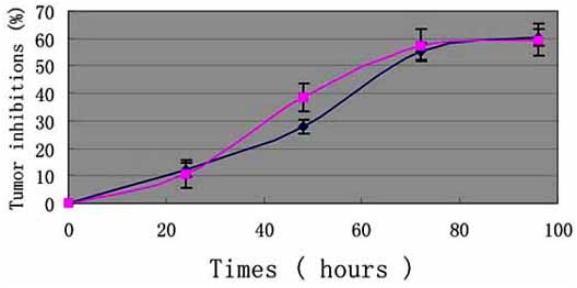

Fig. (4). Time- response curve of probimane on inhibition of a human mammary cell line (MDA-MB-468). MTT method was used. A: Pro concentration 5 μM (dark); B: Pro concentration 0.5 μM (purple).

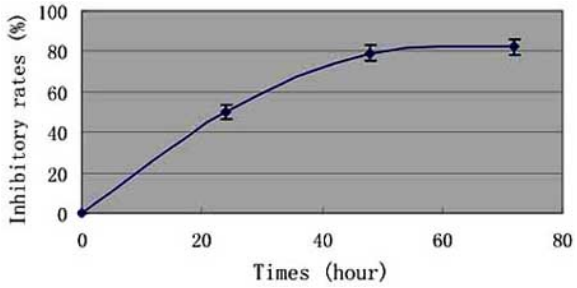

Fig. (5). Time-response curve of probimane on inhibition of a human gastric tumor cell line, SCG-7901. MTT method was used. Pro concentration 5 μM.

the inhibition by Pro (35.7%) was slightly less than that by Raz (40 %) in an 11 day trial. Pro seems to be more persistent than Raz in inhibiting primary LLC tumor growth. The antitumor effects of Raz and Pro on LLC are shown in Tables **7**-**9**. We evaluated 1, 5 and 9 day administration schedules and found that Raz and Pro had statistically significant effects on LLC with a 3-injection regime in all three schedules. If we administered Raz to tumor-bearing mice once on day 1, 5 or 9, there was no difference between treatment and vehicle control. The antitumor effects of Raz in combination with Ble on LLC (73.3 %) were better than

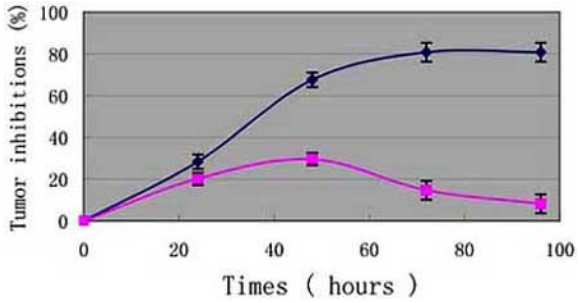

Fig. (6). Time-response curve of probimane on inhibition of a human mammary cell line (MDA-MB-435). MTT method was used.
A: Pro concentration 50 μM (dark); B: Pro concentration 5 μM (purple).

Table 1. The IC_{50} Values of Pro in Different Tumor Cell Lines for 48 h. MTT Method was Used

Cell origin	Cell types	IC_{50} values μM
Gastric	SCG-7901 MKN-28	1.3672 ± 06230 24.314 ± 5.465
Colon	HCT-116	14.476 ± 3.085
Mammary	MDA-MB-435 MDA-MB-468	45.325 ± 5.335 22.169 ± 1.250
Pulmonary	A549	0.02947 ± 0.02456
Leukemia	HL-60 K562	5.3417 ± 1.245 4.786 ± 1.556
Uteric cervical	HeLa	18.238 ± 1.112
Hepatic	BEL-7402	42.457 ± 2.325

Table 2. Cytotoxic Effects of Anticancer Drugs on Tumor Cell Lines *In Vitro*; Drug Exposure for 48 h

Compounds	IC_{50} μM		
	P388	HL-60	HeLa
Doxorubicin	11.7	0.005	1.12
Vincristine	No effect	0.05	4.56
5-fluorouracil	22.6	0.04	0.23
Probimane	64.6	1.97	5.12
ICRF-187	64.0	3.73	129
MST-16	5.23	33.4	26.4

those in combination with Dau (56.3 %) (Table **10**). Pro also showed synergistic effects in combination with Ble.

3. Antitumor Activity of Pro and Raz on LAX-83.

The experiments showed that LAX-83 was sensitive to Raz (40-60 $mgKg^{-1}$, ip×5) and Pro (80-100 $mgKg^{-1}$ ip×5) with 25-32 % and 55-60 % inhibition, respectively ($P<0.01$ *vs* control). CTX, a known anticancer drug (40 $mgKg^{-1}$ ip×5), inhibited the growth of LAX-83 by 84 %. Obvious necrosis in tumor tissues was observed by histological

Table 3. Dose-Response Relationships Among Anti-Cancer Drugs in Respect of Cytotoxicity Against Human Leukemia Line HL-60 (24 h Exposure); * $P < 0.01$; $n = 3$

Compounds	Concentrations	OD values	Percentage inhibition
		Mean ± SD	%
Control	--	1.229 ± 0.125	--
Probimane	10.0	0.298 ± 0.010*	75.6
	2.0	0.260 ± 0.005*	78.9
	0.4	1.142 ± 0.010	7.1
	0.08	1.199 ± 0.012	2.4
Doxorubicin	10.0	0.256 ± 0.021*	79.2
	2.0	0.266 ± 0.013*	78.3
	0.4	0.312 ± 0.016*	74.5
	0.08	0.408 ± 0.031*	66.9
5-Fluorouracil	5.0	0.421 ± 0.021*	65.6
	1.0	0.518 ± 0.012*	57.9
	0.2	0.585 ± 0.025*	54.4
	0.04	0.892 ± 0.038*	27.5
Vincristine	5.0	0.425 ± 0.010*	65.4
	1.0	0.423 ± 0.009*	65.6
	0.2	0.401 ± 0.009*	67.4
	0.04	0.394 ± 0.012*	68.0

Table 4. The Time-Response Relationships Among Different Anticancer Drugs in Respect of Cytotoxicity Against Leukemia Cell Line HL-60; n = 3. Probimane, Pro; 5-fluorouracil, 5-Fu; Doxorubicin, Dox; Vincristine, VCR; (+) Stereo-Isomer of Razoxane, ICRF-187

Compounds	Concentrations	Percentage inhibition %		
	μM	24 h	48 h	72 h
Pro	10	75.6	78.5	75.9
5-Fu	2	65.8	53.5	51.1
Dox	4	78.3	72.4	72.3
VCR	2	65.4	59.0	57.1
ICRF-187	10	47.6	37.8	42.9
MST-16	10	47.8	5.6	0.0

Table 5. The Influence of Pro and Raz on Primary LLC Tumor (Using Student's t-test)

Compounds	Dosage mg/kg/d	Body weight (g)	Tumor weight (g)	Tumor inhibition %
Control	--	23.3 / 24.4	2.80±0.04	--
Razoxane	20	23.3 / 23.4	1.61±0.03*	40.0
Probimane	30	23.4 / 21.6	1.91±0.03*	32.1
Probimane	60	23.3 /23.8	1.80±0.03*	35.7

Route: ip×7 daily. Duration of experiment was 11 days. * P<0.05 (treatment vs vehicle control). The numbers of mice were 30 for the control group and 20 for each treatment group; 100 % survival was observed in each group.

Table 6. The Influence of Pro and Raz on Primary and Metastatic LLC Tumors

Compounds	Dosage mg/kg/d	Body weight (g)	PTI (%)	MFCPM
Control	---	22.8 / 21.4	--	30.9±7.3
Razoxane	20	22.7 / 21.5	40.3	1.2±0.5*
Probimane	30	23.3 / 22.5	42.0	1.5±0.5*
Probimane	60	23.3 / 20.3	48.0	1.0±0.2*

PTI (%) -- Primary tumor inhibition. MFCPM -- metastatic focus count per mouse. Route: ip×7 every 2 days. Duration of experiment was 20 days. * P<0.001 (treatment vs vehicle control). The numbers of mice were 30 for both control group and each treatment group; 100 % survival was observed in each group.

Table 7. Antitumor Effects of Bisdioxopiperazines on Lewis Lung Carcinoma Using Different Schedules

Compounds	Dosage	Schedule	Tumor weight	Tumor inhibition
	mg / kg	1, 5 and 9 day administrations	(g)	%
Control	--	--	2.36±0.05	
Razoxane	80	Once per day	2.49±0.05	-- 5.5
Razoxane	40	Once per day	2.32±0.07	1.7
Razoxane	20	Once per day	2.80±0.06	-- 18.6
Razoxane	10	3 times per day*	1.51±0.04**	36.0
Probimane	20	3 times per day*	1.19±0.05**	49.6

*Administration every 3 hours, 16 mice were included in each testing group. **p < 0.05 (treatment vs control). Duration of experiment was 11 days.

Table 8. Antitumor Effects of Raz on Lewis Lung Carcinoma in Combination with Daunorubicin

Compounds	Dosage	Schedule	Tumor weight (g)	Tumor inhibitions
	mg /kg	1, 5, and 9 day administrations		%
Control			2.34±0.05	
Razoxane (Raz)	10	3 times per day*	1.57±0.05	32.9
Daunorubicin (Dau)	2	Once per day	1.10±0.04	53.0
Raz + Dau	10 + 2	3 times / once per day	1.02±0.04	56.4

*Administration every 3 hours. Duration of experiment was 11 days

Table 9. Antitumor Effects of Raz on Lewis Lung Carcinoma in Combination with Bleomycin

Compounds	Dosage	Schedule	Tumor weight	Tumor Inhibition
	mg /kg	1, 5, and 9 day administration	(g)	%
Control	--	--	2.46±0.06	
Razoxane (Raz)	10	3 times per day*	1.44±0.07	41.5
Bleomycin (Ble)	15	Once per day	1.50±0.06	39.0
Raz + Ble	10 + 15	3 times + once per day	0.66±0.05**	73.2**

Administration every 3 hours in one day. ** $p < 0.01$ (treatment vs vehicle control). Duration of experiment was 11 days.

Table 10. Antitumor Effects of Pro on Lewis Lung Carcinoma in Combination with Daunorubicin or Bleomycin

Compounds	Dosage	Schedule	Body weight	Tumor weight (g)	Tumor inhibition
	mg/ Kg	1, 5, and 9 day administration	g		%
Control	--	--	20.6/ 21.6	2.62±0.08	
Pro	20	Once per day	20.6/ 20.8	1.45±0.07	44.6
Dau	2	Once per day	20.6/ 20.0	1.14±0.08	56.5
Ble	15	Once per day	20.7/ 21.2	1.36±0.08	48.1
Pro + Dau	20 + 2	3 times / once per day	20.6/ 20.9	1.07±0.05	59.2
Pro + Ble	20 + 15	3 times / once per day	20.7/ 19.8	0.59±0.04	77.5

*Administration every 3 hours. Duration of experiment was 11 days.

evaluation of the CTX and Pro treatment groups, but Pro produced larger vacuoles than CTX. Drug effects on tumor volumes were calculated and are summarized in Table **11**. We tested the five most commonly used anticancer drugs: cyclophosphamide (CTX), 5-fluorouracil (5-Fu), methotrexate (MTX), cisplatin (DDP) and vincristine (VCR) (Table **12**). CTX was shown to be most effective in the LAX-83 model. The anticancer effect of Pro against LAX-83 tumor growth was the same as or better than those of MTX, DDP or 5-Fu.

DISCUSSION

The anticancer and anti-metastatic effects and mechanisms of Pro and MST-16 have intrigued us for more than a decade. Increased understanding of the mechanisms of bisdioxopopiperazine action can hopefully widen their indications and narrow their contraindications in clinical practice. Explanations for the anticancer actions of bisdioxopiperazine focus nowadays on anti-angiogenesis [19-20] and effects on tumor DNA rearrangement by topoisomerase II. Generally speaking, most angiogenesis inhibitors have low cytotoxicity

Table 11. Antitumor Activities of Pro and Raz on Human Tumor LAX-83 Using Subrenal Capsule Assay

Compounds	Dosage mg/kg/d	No mice	Body weight (g)	Tumor volume (mm^3)	Inhibition%
Control	---	16	19.2 / 21.0	39.8±3.2	--
Razoxane	40	12	20.8 / 21.5	29.7±3.0*	25
Razoxane	60	12	19.8 / 18.8	27.2±2.8*	32
Probimane	80	12	20.0 / 19.6	18.0±2.6**	55
Probimane	100	12	20.0 / 20.0	15.8±2.6**	60
Cyclophosphamide	40	12	21.0 / 20.9	6.4±2.0**	84

Route: ip×5 daily from the day after surgery. * $P<0.05$, ** $P<0.001$ (treatment vs vehicle control). Experiment was completed within 7 days. Tumor volume = $1/2 \times width^2 \times length$ (using t-test).

Table 12. **Activities of Anticancer Drugs Against Human Tumor LAX-83 Using Subrenal Capsule Assay**

Compounds	Dosage mg/kg/d	No mice	Body weight (g)	Tumor volume (mm^3)	Inhibition%
Control	---	16	20.9 / 22.5	29.7±3.2	--
Methotrexate	1.5	12	21.2 / 21.9	27.4±3.0	7.7
Cis-platin	1.5	12	22.8 / 21.7	16.6±2.6**	44.1
5-fluorouracil	37.5	12	21.7 / 21.4	12.8±2.6**	57.5
Cyclophosphamide	30.0	12	21.0 / 20.9	5.8±2.3**	80.5
Vincristine	0.3	12	20.8 / 20.8	7.6±2.2**	74.4

Route: ip×5 daily from the day after surgery. * P<0.05, ** P<0.001 (treatment vs vehicle control). Experiment was completed within 7 days. Tumor volume = 1/2×$width^2$ ×length (using t-test).

and are ineffective against larger tumor volumes, hence the poor response of patients with large tumor volumes (solid tumors). Clinically, it may be better to combine such agents with cytotoxic drugs [21-22]. The difference in effective dose ranges between Pro and MST-16 can be explained if the cytotoxic effects of Pro and MST-16 are effected through slightly different pathways. Early reports suggest that MST-16 must be transformed into ICRF-154 to exhibit anticancer effects [23]. This work proves that if MST-16 is not changed to ICRF-154 (which, *in vitro*, it is not), it has lower cytotoxic effects against tumor cells than Pro. In future, Pro may be superior in application and require combination with fewer drugs. This work shows that that the activities of Pro against lung cancer and leukemia cell lines are greater than against other tumor categories. Lung cancer constitutes the largest proportion of all cancer categories, and is clinically one of the deadliest cancers. Targeting lung cancers, Pro may be expected to provide greater medical and economic benefits in the future.

To optimize chemotherapeutic protocols involving bisdioxopiperazines, knowledge of the pharmacological parameters related to concentration- and time- responses is a prere-quisite. We found that Pro and MST-16 might act and accumulate in tumor cells for longer than most anticancer drugs. The peak of cytotoxicity for both Pro and MST-16 is on day 3, not on day 2 as for most drugs. This result and our earlier auto-radiographic studies [24] show that Pro persists for longer in tumor tissues, and this suggests that it might help patients by requiring fewer administrations and less nursery care while maintaining high treatment quality. The long-term cytotoxic effects of Pro and MST-16 are more obvious in highly metastatic tumor cell lines. This might explain the selective effects of these compounds on tumor metastases. Since balancing the risks and benefits of a therapy is an important consideration [25- 26], this study provides some of the requisite information.

The anticancer mechanism of Raz is currently attributed to anti-angiogenesis and topoisomerase II inhibition [19- 20, 27]. Since the antimetastatic activities of Raz and Pro are much stronger than their actions against primary tumor growth, this special targeting of metastasis ought to make them more useful in clinical cancer treatment. Our study demonstrates the synergistic anticancer actions of Raz and Pro with Ble or Dau on the basis of this theory. Previous work showed that Pro and Raz reduced the cardiotoxicity of anthrocycline*,* so we may wonder whether they can also reduce the general cytotoxicity of anthrocyclines. Against our expectation, the data in our study suggest synergistic effects between Raz and anthrocyclines, though they are not as potent as those with Ble.

We tested the antitumor activities of five clinically available anticancer drugs (CTX, 5-Fu, MTX, DDP and VCR) against LAX-83, CTX being the best, and found that the two bisdioxopiperazines studied in this work are as effective against tumors overall as these commonly used drugs. Although the anticancer effects of CTX and VCR are better than those of Pro, those of the other commonly used drugs, DDP, MTX and 5-Fu, are not. Since the antitumor effects of MTX and DDP are less than those of Pro and Raz, we suggest that anticancer effects of Pro and Raz are within the effective ranges of the commonly available drugs.

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