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The Anti-Proliferative Effects of Probimane and Razoxane on Tumor Cells are Concomitant with Inhibition of Hemolysis and Calmodulin (CaM) Action and a New CaM-ATPase Acting Model

[1]Da Yong Lu, [2]En Hong Chen, [3]Jing Yi Cao, [2]Jing Bin Xu and [2]Jian Ding
[1]School of Life Sciences, Shanghai University, Shanghai 200444, PR China
[2]Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, PR China
[3]Shanghai Administrative Office, Asahi Chemical Industry Co Ltd, Tokyo, Japan

Abstract: Probimane (Pro), an anti-cancer agent first synthesized in China, is a bisdioxopiperazine derivative. Its parent compound Razoxane (ICRF-159, Raz), was developed in the UK and targets neoplasmic metastases in particular. We have discovered that in addition to the inhibition of metastasis, Pro and Raz have different anti-proliferative effects on tumor cells grown *in vitro*. This finding merits further investigation. In the present study, we have compared Pro and Raz in terms of the relationship between their anti-proliferative effects on tumor cells and their inhibition of hypotonic hemolysis and Calmodulin (CaM) action. We found that Pro decreases red cell lysis by hypotonic saline and inhibits the activity of CaM activated Ca^{++}-Mg^{++}-ATPase in a dose-dependent manner. The differences of anti-proliferative, antihemolytic and anti-CaM effects between Pro and Raz were parallel. Therefore, we propose that the anti-proliferative effects of Pro might operate through CaM inhibition and membrane protection, possibly sialic acid-mediated. Experiments on the effects of these drugs on the dynamics (substrate and time dependence) of red cell membrane CaM-activated Ca^{++}-Mg^{++}-ATPase lead to a new model for their anticancer effects: Pro might be a competitive antagonist of CaM affecting the substrate-product balance of one type of CaM-targeted enzymes-Ca^{++}-Mg^{++}-ATPases.

Key words: Anticancer drugs, bisdioxopiperazine, probimane, razoxane, calmodulin, neoplasm metastases, neuraminic acids

INTRODUCTION

Bisdioxopiperazines, including ICRF-154, Razoxane (ICRF-159, *Raz*), ICRF-186 and ICRF-187 (two stereo-isomers of Raz) and ICRF-193, all developed in the UK, were among the earliest agents found to be effective against a model of spontaneous metastasis (Lewis lung carcinoma) (Herman *et al*., 1982). Since their development (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 (Hellmann and Rhomberg, 1991; Schechter *et al*., 2002), overcoming Multi-Drug Resistance (MDR) to daunorubicin and doxorubicin in leukemia (Sargent *et al*., 2001; Pearlman *et al*., 2003) and inhibiting topoisomerase II (Van Hill *et al*., 2000; Renodon-Corniere *et al*., 2003). More importantly, Raz has been licensed in many countries as a cardioprotectant during anthro-cycline treatment. A considerable volume of research has been published in this area and we do not intend to review it in this paper. Since bisdioxopiperazines (Biz) have distinctly conservative pharmacological actions. Probimane [1,2-bis (N^4-morpholino-3, 5-dioxopiperazine-1-yl) propane; AT-2153, Pro] and MST-16 [1,2-bis (4-isobutoxycarbonyloxy-methyl-3,5-dioxopiperazine-1-yl) ethane] were synthesized at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China (Ji *et al*., 1988; Cai *et al*., 1989). The structural formulae of these three Biz are represented in Fig. 1. MST-16, a licensed drug in Japan since 1994, is licensed for direct use in leukemia chemotherapy, mainly against adult T-cell leukemia (Lu *et al*., 2004, 2005). In addition to data on its anti-tumor activity (Lu *et al*., 2006; Zhang *et al*., 1991, 1993), Pro has been shown to have similar pharmacological mechanisms and actions to Raz, such as amelioration of *Adriamycin* (*ADR*)-induced cardiotoxicity, reported from the Henan Academy of Medicine, China (Yang *et al*., 1990; Braybrooke *et al*., 2000) and other sources (Lu *et al*., 2004). As the principal researchers on Pro, we emphasize the pharmacological and molecular studies of the two compounds in this laboratory.

Corresponding Author: Da-Yong Lu, School of Life Sciences, Shanghai University, Shanghai 200444, PR China

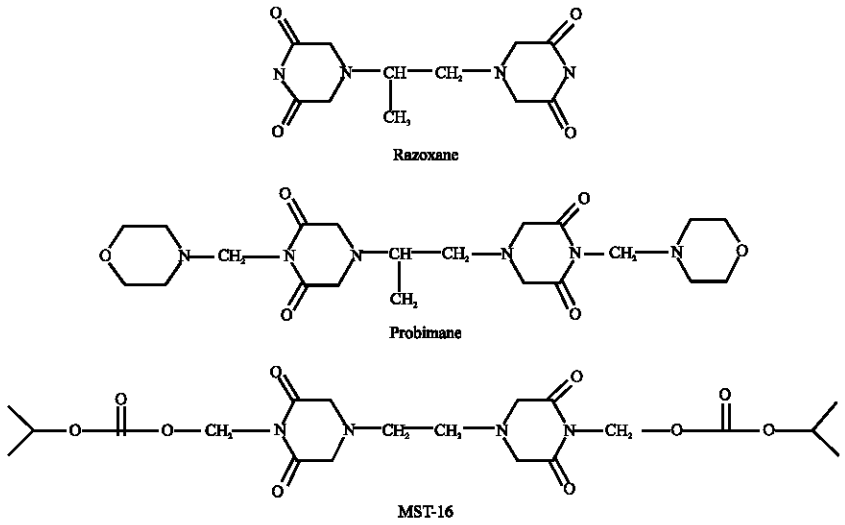

Fig. 1: Structural formulae of razoxane, MST-16 and probimane

Although Biz have been studied for more than four decades, the crucial mechanisms of anticancer action, especially in the case of Raz, remain unclear. The main hypotheses to data have been anti-angiogenesis (Lu *et al*., 1994; Hellmann, 2003), topoisomerase II (Top II) inhibition (Van *et al*., 2000; Renodon-Corniere *et al*., 2003) and prevention of tumor cell detachment from their primary location (Salsbury *et al*., 1974). Anti-angiogenesis is very important for preventing metastases (Taraboletti and Margosio, 2001), but it is a common characteristics of many anticancer drugs: more than 300 compounds are presently known to be angiogenesis inhibitors (Taraboletti and Margosio, 2001; Mark, 2003). The fact that Biz are active against metastasis does not elucidate their exact anticancer pathways, so more specific mechanisms need to be identified. We have previously shown that Pro has greater cytotoxicity (anti-proliferative effect) against solid tumors than Raz *in vitro* (Zhang *et al*., 1993) and *in vivo* (Lu *et al*., 2006; Zhang *et al*., 1993), but the relevant mechanisms have not been identified. The current hypotheses (see above) do not account for the cytotoxicities of these drugs (Zhang *et al*., 1991; Taraboletti and Margosio, 2001). A chance observation in our laboratory revealed that Pro inhibits calmodulin (CaM) activity; CaM, a cell signal regulator, modulates cell function and growth not only in cardiovascular organs (Means *et al*., 1991; Krebs, 1998) but also in neoplasmic (Hait, 1987; Rodriguez-Mora *et al*., 2005; Seiler *et al*., 1998) and other (Wu *et al*., 2001) cells. We therefore proposed that CaM is an anticancer target of Biz, especially of Pro and designed following experiments to assess this hypothesis.

MATERIALS AND METHODS

Drugs and animals: The anticancer agents, Biz agents (Pro and Raz) and α-anordrin were synthesized by the Division of Medicinal Chemistry, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China. Strophanthin G (ouabain) was purchased from Merck Pharmaceutical Co (Germany). Imidazole, histidine, heparin and ATP-2Na were purchased from local biochemical or pharmacological companies. Sialic acid, (N-Acetyl-Neuraminic Acid, NANA) was purchased from Sigma Chemical Co Ltd (MO, USA). The low temperature, high speed centrifuge (PR-52 D) was produced by Hitachi, Japan. The bench centrifuge (80-1) was manufactured by the Shanghai Surgery Instrument Factory, 80-1, Shanghai, China. The spectrometer (Type 752) was manufactured by the Shanghai No. 3 Analytical Instrument Factory.

MTT method: The tumor cells were maintained in RPMI 1640 medium (Gibco, Invitrogen Corporation, NY, USA) supplemented with 10% FCS. Tumor cells were seeded in 96-well micro-plates and incubated for 24 h. The Biz compounds were then added to each well for a further 48 h, then MTT reagent (Sigma Company, MO, USA) (5 mg mL^{-1}, 20 μL) was added to each well. Four hours later, the same volume of 10 % SDS-5% isobutanol-1 N HCl was added and incubated for a further 24 h. The dye absorbance at 570 nm was determined with a tunable microplate reader, VERSAmax, USA.

Rabbit red cells: Blood from healthy male rabbits (2.5-3.5 Kg) was collected into a heparin anti-coagulated tube.

After three washes with normal saline by centrifugation (3000 rpm, 5 min), the red cells were stored for use in an ice bath.

Hypotonic lysis of red cells (hemolysis assay): Rabbit red cells were re-incubated in a 37°C water-bath and hemolyzed for 5 min with hypotonic NaCl (final concentration 0.077 mM) in the presence or absence of Biz. The supernatant (containing hemoglobin from the hemolysate) was quickly pippetted for measurement of the absorbance at 543 nm solution and values were used to assess the degree of membrane protection afforded by the Biz.

Preparation of red cell membranes with active CaM/Ca^{++}-Mg^{++}-ATPase (Wu *et al.*, 2001): Red cells (see above) were lysed with isotonic imidazole buffer (151 mM imidazole 30 μM EDTA, pH 7.4) for 20 min and the membranes were washed three times with hypotonic imidazole buffer (10 mM imidazole, pH 7.5) by high-speed centrifugation (39,000 g, 20 min). All procedures were carried out below 4°C. The washed membranes were stored in a membrane protective solution (40 mM imidazole, 40 mM histidine, pH 7.1) in an ice bath. Under these conditions they remained active for 1 week.

CaM-activated Ca^{++}-Mg^{++}-ATPase assay: Membrane suspensions (0.1 mL containing approximately 350-800 μg membrane protein as quantified by phenol reagent (Farrance *et al.*, 1977) were added along with Biz (0.1 mL) and the reaction mixture (0.1 mL containing 3 mM ATP, 18 mM imidazole, 18 mM histidine, 3 mM $MgCl_2$, 80 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM strophanthin G) to measure enzyme activity. After incubation at 37°C for 1 h, the reaction was abruptly stopped by adding 0.2 mL 10% TCA (trichloracetic acid). Inorganic phosphate (the reaction product) in the supernatants was measured using molybdenum reagent and used as the basis for calculating enzyme activity. The enzyme blank was determined by adding EDTA (0.5 mM) to bind calcium before the reaction started, so no CaM action was detected.

Enzyme dynamics: The enzyme assay system described above was used with following modifications

- The enzyme substrate (ATP-2Na) concentrations were 3 and 6 mM
- Reactions were allowed to continue for 1, 5 min and 1 h;
- Antagonist concentrations were varied. The time- and substrate- response curves were plotted separately.

RESULTS

Comparison of anti-proliferative effects of Pro and ICRF-187: The anti-proliferative effects (IC_{50} values) of three Biz against two human mammary tumor cell lines MDA-MB-435 and MDA-MB-468 were determined after 48 h co-culture. Pro, MST-16 and ICRF-187 showed moderate *in vitro* cytotoxicity against both cell lines. Pro (IC_{50}, 4-20 μM) was relatively more effective than ICRF-187 (IC_{50}, 30-250 μM). Table 1 and 2; Fig. 2 and 3. This provides a basis for the following studies.

Membrane protection by Pro as determined by hemolysis assays *in vitro*: Pro (60 μM) significantly decreased the rate of hemolysis of rabbit red cells in hypotonic saline from 47.0% to 42.0% (inhibition 10.6%, $p<0.01$). Raz at 60 μM afforded less effective cell membrane protection (inhibition 3.6%, NS). Table 1 shows that the membrane protection effect of Pro is about 3-fold higher than that of Raz at equivalent concentrations. (Table 3)

Table 1: Antiproliferative effects of bisdioxopiperazines against human colon carcinoma HCT-116 *in vitro*

Compounds	Concentrations μLM	OD±SD	Inhibition %
Control		0.978±0.086	
Probimane	100	0.539±0.071*	44.8
	10	0.914±0.012	6.6
	1	0.942±0.018	3.7
Razoxane	100	0.866±0.110	11.5
	10	0.908±0.076	7.1
	1	0.939±0.055	4.0
MST-16	100	0.795±0.025	18.7
	10	0.866±0.030	11.5
	1	0.935±0.028	2.0

*$p<0.05$

Table 2: Anti-proliferative effects of bisdioxopiperazines against human mammary tumor cell line MDA-MB-468 cell line *in vitro*

Compounds	Concentrations μLM	OD±SD	Inhibition %
Control		1.336±0.086	
Probimane	100	0.452±0.012**	66.1
	10	1.115±0.022**	16.5
	1	1.241±0.028	7.1
Razoxane	100	1.069±0.033**	20.0
	10	1.153±0.018*	13.6
	1	1.235±0.030	7.5
MST-16	100	1.113±0.025**	16.7
	10	1.235±0.019	7.5
	1	1.313±0.018	1.7

Table 3: The protective effects of bisdioxopiperazines against hemolysis by hypotonic saline (0.077 mM NaCl); n = 3, ** $p<0.01$

Compounds	Concentrations μM	Relative hemolyses	inhibition %
Control	--	47.0±0.8	--
Probimane	30	44.9±1.0	4.5
	60	42.0±0.6**	10.6
Razoxane	30	45.4±0.5	3.4
	60	45.3±0.7	3.6
MST-16	60	46.0±0.6	2.2

Fig. 2: Anti-proliferation effects (IC_{50}) of probimane and razoxane against human mammary tumor cell line (MDA-MB-435); MTT assay for 48 h.

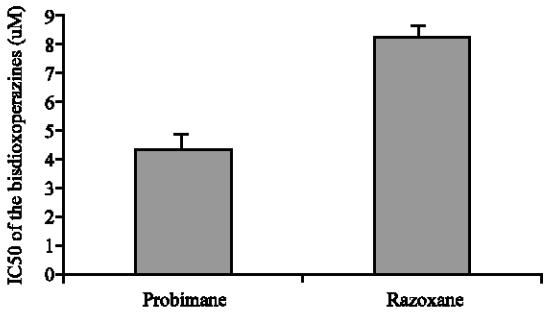

Fig. 3: Anti-proliferation effects (IC_{50}) of probimane and razoxane against human leukemia cell line (K562); MTT assay for 48 h.

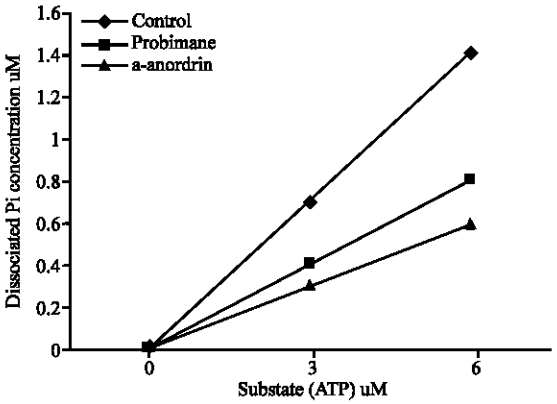

Fig. 4: Substrate (ATP)-response curve. Probimane (30 μM) and α-anordrin (10 μM) on membrane solution (CaM and Ca^{++}-Mg^{++}-ATPase) with different substrate (ATP) solution (1:50 v/ v) for 1 h (n= 3)

Inhibition of CaM-activated Ca^{++}-Mg^{++}-ATPase: This experiment showed that Pro (> 0.1 mM) significantly inhibits the activity of CaM-activated Ca^{++}-Mg^{++}-ATPase in rabbit red cell membrane (inhibition>11.4%, $p<0.001$)(Table 4). However, Raz and MST-16 at 0.5 mM give only 2% inhibition (Table 4). The CaM-activated Ca^{++}-Mg^{++}-ATPase activity is gradually decreased during the storage of the membranes. After 1 week, the CaM-activated enzyme activity decreased from 83.5 $\mu MPimin^{-1}g^{-1}$ $protein^{-1}$ to 60.7 $\mu Mpimin^{-1}g^{-1}$ protein. Pro at 0.2 mM had no statistically significant inhibitory effect on CaM-activated enzyme in the week-old membranes. However, after the addition of sialic acids (NANA), significant inhibition was obtained with 20 to 100 μM Pro (Table 5); also Pro 0.1 mM was significantly inhibitory in membranes stored for 4 d even in the absence of sialic acid. This again suggests that Pro is a more effective inhibitor of CaM- activated Ca^{++}-Mg^{++}-ATPases than Raz at equivalent concentrations.

Table 4: Effects of Pro and Raz on CaM-activated Ca^{2+}-Mg^{2+}-ATPase of rabbit erythrocyte membranes. Inhibitory rates = $EA_{control}$-EA_{drug} / $EA_{control}$-EA_{EDTA}, n = 3, *** $p<0.001$ compared with control

Compounds	Concentrations μM	Ca^{2+}-Mg^{2+}-ATPase Enzyme activity (EA)	Inhibition %
Control	--	83.5±0.8	
Pro	1000	64.2±1.7***	32.2
	400	75.2±1.7***	13.9
	100	76.7±1.0***	11.4
Raz	500	81.8±1.5	2.0
MST-16	500	82.2±1.7	--
EDTA	500	23.5±1.7	

Table 5: Synergism of Pro and N-acetylneuraminic acid (NANA) on CaM-activated Ca^{2+}-Mg^{2+}-ATPase of rabbit erythrocyte membrane. Inhibitory rates = $EA_{control}$-EA_{drug} / $EA_{control}$-EA_{EDTA}, n = 3, ** $p<0.01$ compared with control

Compounds	Concentrations μM	Ca^{2+}-Mg^{2+}-ATPase Enzyme activity (EA)	Inhibition %
Control	--	60.7±2.3	
NANA	200	57.3±0.0	8.9
Pro	200	61.2±0.0	--
NANA+Pro	200+100	50.8±1.5**	26.5
NANA+Pro	100+20	54.7±0.7**	16.1
EDTA	500	23.5±0.8	--

The differences of anti-proliferative, antihemolytic and anti-CaM effects between Pro and Raz are parallel: The above data suggested that the differences of anti-proliferative, antihemolytic and anti-CaM effects between Pro, Raz and MST-16 are parallel suggesting that the mechanisms of action of these two Biz derivatives are similar. This novel discovery is important for identifying the pharmacological targets of Biz.

Dynamic study of CaM-activated Ca^{++}-Mg^{++}-ATPase: The data in Fig. 4 (substrate-response curve) indicate that the effects of the CaM inhibitors examined, Pro and α-anordrin, were unchanged when the ATP (substrate) concentration was varied (3 or 6 mM) (Fig. 4). The increase in inorganic phosphate formation with increasing substrat concentration was in the same ratio in both treatment groups and in the control groups (no inhibitor)

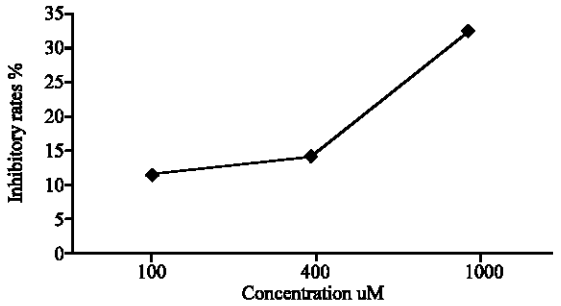

Fig. 5: Dose- response curve of low concentrations of probimane addition to CaM activated Ca^{++}-Mg^{++}-ATPase activity

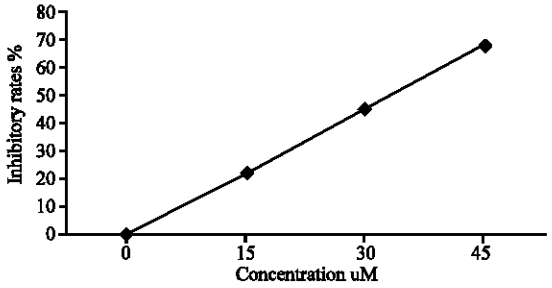

Fig. 6: Dose- response curve of α-anordrin addition to CaM activated Ca^{++}-Mg^{++}-ATPase activity

suggesting that the drugs in the concentration used only affect the substrate-product balance produced by the coupled enzymes in the red cell membrane. However, different inhibitor concentrations at the same CaM concentration (maintaining constant membrane resource), have different effects on Pi formation compared to controls. The dose-response relationships are given in Fig. 5 and 6. More unexpectly, we found that the substrate-product balance is the same after 1, 5 min and 1 h incubations irrespective of inhibitor (Pro or α-anordrin) and ATP concentrations. This indicates that the enzyme-catalyzed reaction is rapid and stable over relatively long intervals. Thus, we propose a competitive inhibition model for the CaM-inhibitors (Pro and α-anordrin); Pro and α-anordrin inhibitions maintain the ratio of ATP to free phosphate but do not significantly alter the catalytic rate of the enzyme. This is a novel explanation for the action of these drugs.

DISCUSSION

The anticancer mechanisms of Biz remain unknown. The effects of bio-active compounds on erythrocyte membrane stabilization and CaM inhibition are often correlated (Bereza *et al*., 1982) and anti-CaM agents were the earliest types shown to have cardiovascular-regulatory effects (Means *et al*., 1991; Krebs, 1998). This pharmacological property has now been extended to some anticancer (Rodriguez-Mora *et al*., 2005; Seiler *et al*., 1998) and the other (Wu *et al*., 2001) drugs. In the present study, we sought to explore the hypothesis that different cytotoxic (anti-proliferation) effects of Biz agents might be related to differences in their anti-CaM effects. Our evidence in favor of this pathway inhibition suggests that Pro might have such a pharmacological role. Although this hypothesis seems to derive directly from previous knowledge, the correlation of the effect with sialic acids (NANA) indicated by the present results is new. Since 1980, it has been recognized that sialic acids correlate with tumor growth and metastasis (Yogeeswaran *et al*., 1981; Lu and Cao, 2001; Lu *et al*., 1994). We have found that Pro reduces the sera sialic acid levels in mice bearing solid tumors (Lu *et al*., 1994). This work consolidates the proposed links between sialic acids, CaM-mediation and Pro, as an anti-metastatic agent. CaM inhibitors have previously been suggested to overcome Multi-Drug Resistance (MDR) in tumor cells (Nair *et al*., 1986). Cytotoxic data on Pro support this (Zhang *et al*., 1994).

More unexpectedly, the enzyme dynamic study implied a new model for antagonism of CaM-activation. According to this model, the effect of Biz on signal transduction through CaM is responsible for their pharmacological effects, targeting enzymes down stream of CaM such as Ca^{++}-Mg^{++}-ATPases. Pro and α-anordrin, as strong long-term competitive antagonists of CaM could bind covalently or non-covalently to alter the form and activity of targeted enzymes Ca^{++}-Mg^{++}-ATPases or others (Means, 2003; Krebs, 1998; Hait, 1987; Rodriguez-Mora *et al*., 2005; Seiler *et al*., 1998; Wu *et al*., 2001) thus determining cell growth or survival rates. α-anordrin is another anticancer drug `discovered in Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Weng *et al*., 1994; Lou and Xu, 1996) and was used for comparison here in exploring and explaining our hypothesis. This model of CaM-drug-enzyme interaction has not been proposed previously and might initiate further research. However, by this experiment can only explain enzyme model of CaM-ATPase system. We can not draw conclusion of CaM activated into other enzymes or cell signal molecules.

To conclude; since Pro and Raz have parallel anti-proliferative, antihemolytic and anti-CaM effects, we conclude that Pro protects rabbit red cells against hypotonic lysis and inhibits CaM-activated Ca^{++}-Mg^{++}-ATPase activity and antagonizes cell proliferation more strongly than its parent compound, Raz. This is the pharmacological significance of Biz. According to our proposed model, Pro and á-anordrin are CaM competitors

with CaM-like binding and their action maintains the substrate-product balance of CaM activated Ca^{++}-Mg^{++}-ATPases.

CONCLUSION

Therefore, we conclude that the anti-proliferative effects of Pro might operate through CaM inhibition and membrane protection, possibly sialic acid-mediated. Experiments on the effects of these drugs on the dynamics (substrate and time dependence) of red cell membrane CaM-activated Ca^{++}-Mg^{++}-ATPase lead to a new model for their anticancer effects: Pro might be a competitive antagonist of CaM affecting the substrate-product balance of CaM-targeted enzymes such as Ca^{++}-Mg^{++}-ATPases.

List of abbreviations used are: CaM, calmodulin; Pro, probimane; Raz, razoxane, ICRF-186, ICRF-187; LPO, lipoperoxidation; Biz, bisdioxopiperazine compounds £»TOPII, topoisomerase II

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