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The interaction of platinum and anthrapyrazole antitumor drugs with mouse thymocytes studied by terbium fluorescence

H. Simpkins, Maria Figliomeni and M. Rosen

Departments of Pathology, State University of New York, Health Sciences Center, Downstate, New York and the Staten Island Hospital, New York and the Department of Biology, City University of New York, Staten Island, NY (U.S.A.)

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The fluorescent lanthanide, terbium has been employed to study the effect of a series of platinum and anthracycline drugs and an anthrapyrazole (oxanthrazole) on terbium binding to mouse thymocytes. It was observed that terbium fluorescence intensity was markedly decreased by two platinum drugs (*cis*-dichlorodiammine platinum(II) (*cis*-DDP) and *cis*-dichloro-*trans*-dihydroxybis(isopropylammine) platinum(IV) (CHIP)) and an anthrapyrazole (oxanthrazole), but that the lipophylic derivative *cis*-diammine-1,1-cyclobutanedicarboxylate platinum(II) had a small but significant effect and the anthracyclines (at low concentrations) had no effect. The calcium channel blocker, verapamil also had no effect. The effect of *cis*-DDP was markedly dependent on ionic strength in contradistinction to CHIP. The decreases in phosphorescence decay produced by *cis*-DDP also showed a marked dependence on ionic strength. It is proposed that *cis*-DDP interacts with the membrane primarily by a charge effect, but that CHIP may produce a conformational change in the membrane. These data are interesting, since the lipophylic platinum drugs (CHIP and CBDCA) also increased significantly the amount of bound intracellular calcium, but all the drugs decreased mitogen-stimulated calcium uptake into mouse thymocytes.

Introduction

One of the most widely used antitumor drugs presently in use is *cis*-DDP which is used to treat a whole range of solid tumors of various origins [1–6]. Unfortunately its therapeutic efficacy is limited by severe dose-limiting side effects, the prime one being nephrotoxicity [7] and thus, a series of second generation platinum derivatives have been synthesized in an attempt to find derivatives with less side effects than the parent compound. Two of these second generation platinum drugs include CBDCA (JM-8) and CHIP (JM-9). CBDCA was found to be potent against plasmacytoma PC6A, human epidermoid P246 and L1210 leukemia cell lines and CHIP was effective against the L1210 and the PC6A cell line, but had little effect on an epidermoid carcinoma line [8]. The side effects in animal models were minimal compared to the parent compound. In recent clinical trials, CBDCA appeared to produce remission

Abbreviations: *cis*-DDP, *cis*-dichlorodiammine platinum(II) CBDCA, *cis*-1,1-cyclobutanedicarboxylate platinum(II) CHIP, *cis*-dichloro-*trans*-dihydroxybis(isopropylammine) platinum-(IV) AD-32, *N*-trifluoroacetyladriamycin 14-valerate; MRA-CN, 3′-deamino-3′-(3-cyano-4-morpholinyl)doxorubicin; TMA-DPH, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene-*p*-toluene sulfate; MOPS, 4-morpholinepropanesulfonic acid; Heps, *N*-2-hydroxyethylpyrazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline.

Correspondence: H. Simpkins, Department of Pathology, State University of New York Health Sciences Center, Downstate, New York, NY 11203-2093, U.S.A.

rates comparable to those of *cis*-DDP in advanced ovarian as well as breast carcinoma [9,10] and CHIP appeared to have a response rate in advanced ovarian and breast carcinoma similar to that reported with *cis*-DDP, but its main side effect was myelosuppression [11,12]. The anthrapyrazoles are a group of tumor compounds presently under study. They have been shown to be less cardiotoxic, employing the cultured mouse heart model, than adriamycin, but to possess a similar antitumor effect in animal models [13].

Calcium channel blockers, such as verapamil, have recently been shown to potentiate the antitumor effect of *cis*-DDP on human neuroblastoma cells [14]. The properties of the calcium channels in *cis*-DDP sensitive L1210 cells were also reported to differ from those in the resistant cell line [15]. Resistance to the anthrapyrazoles can be reversed by verapamil [16], suggesting that calcium channels may play an important role in the mechanism of action of these drugs in much the same way as has been implicated for these channels with the anthracyclines (for review see Kessel [17]).

The terbium ion (Tb^{3+}) has been used as a fluorescent probe for calcium-binding sites with a series of calcium-binding proteins [18,19]. It was shown that terbium does not enter intact living cells, but binds to their outer membrane surface [20,21]. This cation has also been used to map the distribution of calcium-binding sites on cultured neurons [22]. Terbium has also been employed to study the calcium-binding sites on human platelets and how these sites are perturbed by treatment with ADP [23]. It has been used recently to investigate the effects of *cis*-DDP binding to GH3/B6 pituitary tumor cells [24] and, from these results, a specific receptor for *cis*-DDP was proposed. It is assumed that the increase in terbium fluorescence, when interacting with calcium-binding sites is due to energy transfer between the terbium ion and the surrounding tyrosine and tryptophan residues, although in mitochondria this energy exchange may also involve the heme groups of the cytochromes. Terbium has also been used as a probe of the surface membrane potential of the mitochondrial membrane and its calcium-binding sites [25]. The results in this paper outline studies using terbium fluorescence as a probe of calcium channels on the surface membrane of the mouse thymocyte and how these are altered by platinum, anthrapyrozole and adriamycin antitumor drugs. Thymocytes were chosen for the initial studies, since the effect of platinum drugs on their membrane lipids has been investigated [26], they can be readily stimulated to divide by mitogens in a well-defined manner and this process has been shown to be accompanied by a marked increase in intracellular Ca^{2+} levels [27]. Thus, the work was continued by correlating the effect of these drugs and verapamil on calcium uptake by mouse thymocytes in their resting state as well as when stimulated by a mitogen.

Materials and Methods

cis-DDP, CHIP, CBDCA, AD-32, MRA-CN and oxanthrazole were obtained from the drug synthesis and chemistry branch of the National Cancer Institute, Bethesda, MD. The drugs were used without further purification. TMA-DPH and Indo-1 AM ester were purchased from Molecular Probes, Inc. (Eugene, OR)

Preparation of thymocytes. Thymus glands from Balb/c mice (average weight 12 g) were surgically excised. A single-cell suspension was prepared by either: (a) teasing the tissue gently between the frosted ends of two glass microscope slides or, (b) rubbing the tissue through a stainless steel sieve with a mesh size of 180 μM (ATM, Milwaukee, WI) into PBS solution. The cells were washed twice in the same buffer by centrifugation at 400 $\times g$ for 10 min and then resuspended in the same buffer at a concentration of $(2–5) \cdot 10^7$ cells/ml. The cells were counted in a hemocytometer on the basis of viability as tested by Trypan blue exclusion. Viability was consistently greater than 95%.

Estimation of terbium binding to the thymocytes. The binding of terbium to the thymocyte suspension was estimated by measuring the free concentration of terbium employing dipicolinic acid as a luminescence sensitizer [28]. The thymocyte suspension was incubated with terbium at concentrations of 0–300 μM, centrifuged at 400 $\times g$ for 3 min and then reacted with the dipicolinic acid at a final concentration of 400 μM. The fluorescence was measured at an excitation wavelength of 280 nm and an emission wavelength of

545 nm. A calibration curve was constructed using a standard $TbCl_3 \cdot 6H_2O$ solution which had been calibrated with EDTA [29]. The thymocyte protein concentration was determined according to Lowry et al. [30] employing bovine serum albumin as a standard.

Terbium fluorescence and phosphorescence decay studies. Increasing concentrations of terbium were added to the cell suspension (0–150 μg of protein) in either sucrose-Mops buffer (0.25 M sucrose/1 mM Mops, pH 7.4) or KCl-Mops buffer (0.145 M KCl/1 mM Mops, pH 7.4). The fluorescence spectra were measured using an MPF43A fluorescence spectrophotometer with a high-pressure xenon lamp. An excitation wavelength of 290 nm and emission wavelength of 544 nm were employed to maximize energy transfer and minimize light scatter. The entrance and exit slit-widths were kept at 5 nm which was important to minimize light scatter. The appropriate blanks were used for the cell suspensions as well as the drug solutions.

The thymocytes were labeled with TMA-DPH by making a stock solution of 5 mM of the probe in dimethyl formamide. This solution was diluted to 0.04 mM in the appropriate buffer by vigorous agitation. The diluted probe was then reacted 1 : 1 with 150 μg of cell protein/ml at room temperature for 15 min. The fluorescence intensity was measured at 37 °C (in a jacketed cuvette) with polarization filters at 0–0°, 90–0°, 0–90° and 90–90°. The optimum excitation and emission wavelengths were 358 and 440 nm, respectively. The slit-widths were kept constant 5 nm. The polarization constant P was calculated from the formula

$$P = \frac{V_v - L_v(V_H/L_H)}{V_v + L_v(V_H/L_H)}$$

where V_v is the fluorescence intensity at 0–0°; V_H, 90–0°; L_H, 90–90°; L_V, 0–90°.

Phosphorescence decay curves were obtained with a Perkin Elmer LS5 luminescence spectrophotometer. Excitation was by a repetitive discharge of the lamp with a half-width duration of 10 ms. The phosphorescence decay curve was measured varying the delay time (0.02 ms to 2 ms) with a fixed gate time (7 ms) with automated air blank subtraction.

Calcium influx studies. Fresh thymocytes were labeled with Indo-1 by incubation of 150 μg of cellular protein/ml PBS or dye and bicarbonate-free RPMI-1640 medium containing 0.5 mM $CaCl_2$ and 5 μM Indo-1/AM ester at 37 °C for 30 min (the Indo ester was added as 5 μl of a 1 mM stock solution in dimethyl sulfoxide) together with the different drugs [27]. The fluorescence of the bound calcium was read at 405 nm and that of the free was read at at 485 nm. The excitation wavelength was 355 nm. Concanavalin A was added at a concentration of between 10 and 30 μg/ml and the fluorescence was monitored over 6 min at both wavelengths to determine the effect of drug treatment on mitogen-stimulated calcium uptake.

Results

The terbium fluorescence intensity was stable when reacted with control thymocytes; however, in contradistinction from GH3/B6 pituitary cells [24] and rat liver mitochondria [25], saturation was not totally achieved at Tb^{3+} concentrations of 300 μM with 100–150 μg of total cellular protein. The observation that the terbium fluorescence intensity is stable with time is important, since it implies that there is little terbium transport into the cells as the high intracellular concentration of phosphate would result in terbium precipitation and, thus, the fluorescence intensity would decrease with time. Data is presented in Table I showing that a decrease in terbium fluorescence intensity is produced by treatment with some platinum drugs and an anthrapyrazole (oxanthrazole). It is apparent that *cis*-DDP, CHIP and oxanthrazole produce a marked effect and CBDCA produces a small but significant decrease in terbium fluorescence. The effect of ionic strength on terbium fluorescence is also different for the two platinum drugs (Fig. 1). The effect of ionic strength is minimal for CHIP but is marked with *cis*-DDP and, thus, is similar to the effect observed when these drugs are reacted with the mitochondrial membrane (Rosen, M. et al., unpublished results). However, when a whole series of adriamycin derivatives (adriamycin, AD-32, MRA-CN, daunomycin and actinomycin) were reacted at low drug concentrations (to avoid quenching) with the thymocytes little effect was

TABLE I

EFFECT OF PLATINUM AND OXANTHRAZOLE ANTITUMOR DRUGS ON THE FLUORESCENCE INTENSITY OF TERBIUM REACTED WITH MOUSE THYMOCYTES

The experimental procedure is described in the Materials and Methods employing a 0.145 M KCl-Mops (pH 7.4) buffer. The terbium fluorescence intensity of the non-treated thymocytes was arbitrarily set at 100%. Values are expressed ±S.E. and the numbers in parenthesis refer to the number of experiments.

Drug treatment	Terbium fluorescence intensity
None	100%
100 μM *cis*-DDP	82±10 (4)
200 μM *cis*-DDP	45±10 (6)
200 μM CHIP	40± 8 (6)
100 μM CBDCA	88± 6 (2)
200 μM CBDCA	82± 8 (4)
5 μM oxanthrazole	50±10 (4)
50 μM verapamil	100± 5 (4)

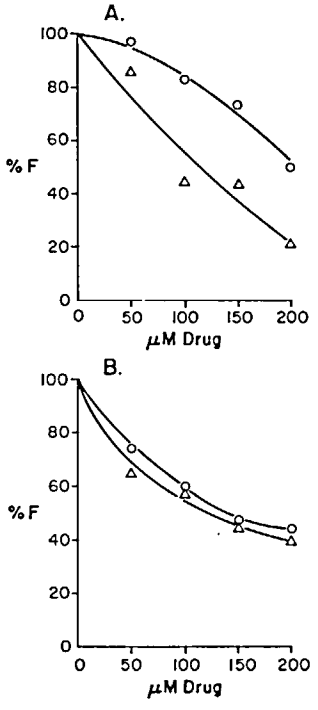

Fig. 1. The effect of *cis*-DDP (A) and CHIP (B) on the terbium fluorescence intensity of mouse thymocytes studied in high (○———○) and low ionic strength buffers (△———△). The terbium concentration was kept constant at 300 μM and the total cellular protein concentration was maintained at 150 μg. It is apparent that little effect of ionic strength is observed in panel B compared to panel A.

produced on terbium fluorescence intensity (95–106%: control arbitrarily set at 100%).

The phosphorescence decay curve of terbium interacting with mouse thymocytes is presented in Fig. 2. The decay curve of the non-treated cells can be analyzed by a two-site model, one site with high affinity and a rapid decay rate and one with low affinity and a slow rate of decay. If the decay curves in high and low ionic strength are measured, the surface potential can be calculated, as was recently reported for the mitochondrial membrane [25]. Such a detailed analysis is difficult to perform with these cells, since the phosphorescence intensity is much lower than that observed with the mitochondrial membrane and, thus, relatively high saturating Tb^{3+} (300 μM) concentrations were used to obtain the results, and such high concentrations of Tb^{3+} will alter the measured membrane surface potential. However, when the decay curves of the drug-treated cells are analyzed, *cis*-DDP produced a markedly different effect as compared to that produced by CHIP (Fig. 2A and B). *cis*-DDP produced a minimal effect on the decay rate of the slow phase (low affinity site) in high salt, but a marked effect was produced by the drug in a low ionic strength buffer. In contradistinction, CHIP produced a far greater decrease in the decay rate but the effect was not markedly dependent on ionic strength. These results parallel those observed with fluorescence intensity measurements where a marked effect of ionic strength was observed on the decrease in fluorescence intensity produced by *cis*-DDP. These results suggest that *cis*-DDP probably acts via a charge–charge interaction, whereas CHIP may alter the conformation of the cell membrane producing a decrease in energy transfer of the Tb^{3+} to the protein tryptophan residues, a hypothesis not unreasonable in view of results with lipid probes showing that CHIP but not *cis*-DDP produced a marked effect on mouse thymocyte lipid domains [26].

Terbium binding/uptake was also determined with the intact thymocytes and it was was found (see Table II) that approx. 93% and 80% of the Tb^{3+} was associated with the cells in the high and low ionic strength buffers, respectively, and that *cis*-DDP and CHIP treatment produced a small but significant increase in the low ionic strength

buffer but a negligible effect in high concentrations of salt. Fluorescence measurements paralleled the binding studies, since it was found that

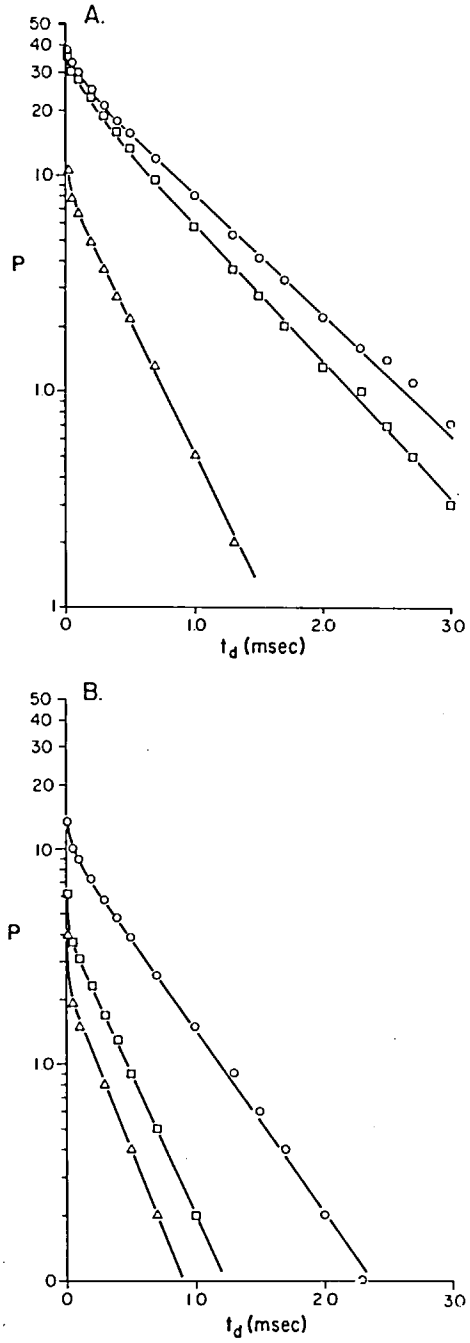

TABLE II

TERBIUM ASSOCIATION WITH MOUSE THYMOCYTES FOLLOWING TREATMENT WITH PLATINUM AND OXANTHRAZOLE DRUGS

The thymocytes were treated as described in the Materials and Methods by measuring the free Tb^{3+} in the supernatant with dipicolonic acid as a luminescence sensitizer. The association is expressed as Tb^{3+} bound/Tb^{3+} total. Values are expressed ±S.E. and the numbers in parenthesis are the number of experiments.

	0.145 M KCl-Mops (pH 7.4)	0.25 M Sucrose-Mops (pH 7.4)
Control (300 μM Tb^{3+})	0.97±0.02 (5)	0.80±0.06 (6)
90 μM *cis*-DDP	0.96±0.04 (3)	0.90±0.03 (3)
90 μM CHIP	0.97±0.05 (3)	0.90±0.05 (3)
200 μM *cis*-DDP	0.95±0.04 (4)	0.90±0.03 (4)
200 μM CHIP	0.94±0.06 (4)	0.88±0.05 (4)
5 μM oxanthrazole	0.99±0.01 (4)	0.83±0.06 (4)

the terbium fluorescence intensity of thymocytes was markedly greater (65–75%) in a high ionic strength buffer.

In order to determine whether the drugs produce a marked effect on calcium transport through the plasma membrane of the thymocyte, the effect of the drugs on intracellular calcium binding and mitogen-stimulated calcium uptake was studied. The mitogen employed was a polyvalent one – concanavalin A and the results of such a study showed (see Table III) that the intracellular bound Ca^{2+} concentrations were markedly increased by drug treatment, CHIP and CBDCA having the greatest and oxanthrazole, verapamil and adriamycin, the least effect. However, all drugs de-

Fig. 2. The decay in terbium phosphorescence when reacted with control mouse thymocytes (○———○) and following treatment with *cis*-DDP (□———□) and CHIP (△———△) in high (A) and low ionic strength buffers (B). The curves generated from the control thymocytes can be analyzed by a two-component model. It is evident that treatment with *cis*-DDP causes a minimal effect in high ionic strength (A), but a marked effect at low ionic strength (B), whereas CHIP produces a marked effect in both high and low ionic strength buffers.

TABLE III

EFFECT OF ANTITUMOR DRUGS ON BASAL AND MITOGEN-STIMULATED CALCIUM UPTAKE BY THYMOCYTES

The calcium uptake is expressed as the ratio of Ca^{2+} bound/Ca^{2+} free calculated from the ratio of the fluorescence intensities as determined with the Indo-1/AM ester at 405 and 485 nm with excitation at 355 nm. Values are expressed ±S.E. and the numbers in parentheses refer to the number of experiments.

Drug treatment	Ca^{2+} bound/ Ca^{2+} free	% Increase following mitogen treatment
Control	0.73±0.06 (7)	35±6 (7)
100 μM *cis*-DDP	0.73±0.05 (6)	31±7 (4)
200 μM *cis*-DDP	0.94±0.08 (5)	21±6 (3)
100 μM CHIP	0.98±0.08 (6)	18±8 (4)
200 μM CHIP	1.13±0.10 (8)	8±4 (6)
1 μM oxanthrazole	0.76±0.04 (7)	18±5 (5)
5 μM oxanthrazole	0.80±0.07 (5)	13±2 (3)
200 μM CBDCA	1.15±0.08 (3)	8±4 (3)
10 μM verapamil	0.85±0.10 (3)	15±5 (3)
1 μM adriamycin	0.85±0.10 (2)	14±5 (3)

creased mitogen-stimulated uptake including oxanthrazole, *cis*-DDP having the least effect.

Discussion

Two sites have been proposed for plasma membrane calcium channels, one of which is thought to lie at the outer mouth of the calcium channel and the second near the inner mouth [31]. Organic calcium channel blockers, such as verapamil, are thought to interact at site 2 presumably via a lipophylic interaction, whereas inorganic calcium channel blockers (lanthanide and Cd^{3+}) are thought to act at site 1. Thus, terbium would presumably interact at site 1, but verapamil at a different locus. The data presented here show that two platinum drugs and an anthrapyrazole have marked effects on the terbium-binding sites on the thymocyte plasma membrane. The results also show that a whole range of anthracyclines at low drug concentration (1 μM) do not produce any marked effect. The lipophyllic platinum drug CBDCA had a small but significant effect. The relative lack of effect of the anthracyclines and CBDCA on the Tb^{3+} site (site 1) is not an unexpected finding, since these drugs have an effect on plasma membrane lipid fluidity [26,32,33] and, thus, presumably interact primarily on the lipophylic Ca^{2+} channel (site 2). It is interesting to speculate on the negligible effects of the adriamycin derivatives on the terbium-binding sites in the plasma membrane, since these compounds were shown to produce significant effects at this site at these low concentrations on the mitochondrial membrane (Rosen, M. et al., unpublished results). It is known that the mitochondrial membrane is rich in cardiolipin and that adriamycin reacts specifically with cardiolipin to form a drug-lipid complex [34]. Thus, it appears likely that the terbium-binding site, on the mitochondrial membrane involves this lipid (which is also a target for the anthracyclines). Since this lipid is not found in the plasma membrane, little effect would be expected when adriamycin interacts with the plasma membrane, a hypothesis which correlates with the observations presented here with mouse thymocytes. A recent article by Canada et al. [35] reports data with GH3/B6 pituitary tumor cells interacting with adriamycin at higher drug concentrations (3–50 μM). These authors show quenching between bound and free drug and the Tb^{3+} site, suggesting interaction of both bound and free drug with Tb^{3+} at these higher drug concentrations.

The effects of the two platinum drugs on the thymocyte membrane are quite different. *cis*-DDP produces an effect on terbium fluorescence which appears quite dependent on ionic strength, a result which correlates to its effect on terbium phosphorescence decay where its effect on the slow component appears to be markedly dependent on ionic strength in contradistinction to CHIP and oxanthrazole.

Finally, it is interesting to note that verapamil has little effect on terbium fluorescence even at relatively high drug concentrations, in contradistinction to the results with the mitochondrion, where a significant effect was observed. These observations are in keeping with suggestions relating to the plasma membrane calcium site/channels, where two sites are proposed. The organic calcium channel blockers, such as verapamil, are thought to effect site 2, presumably by a lipophylic interaction. These results support the idea that the terbium site correlates to site 1,

whereas lipophylic substances, such as CBDCA and verapamil, interact with site 2 and, thus, have little or no effect on terbium binding and its fluorescence. CHIP is more complex, since it produces an effect on thymocyte membrane fluidity [26] as well as terbium fluorescence and, thus, may effect both sites – a not unreasonable idea, since this compound consists of both highly charged and lipophylic side groups.

One point that must be addressed is whether the decrease in terbium fluorescence produced by the drugs could be explained by an increased inward transport of terbium into the cell and precipitation by the high intracellular concentrations of phosphate. The results of such studies showed that most of the terbium was associated with the cells and little change was observed following drug treatment, except at low ionic strength where a small but significant effect was observed with *cis*-DDP and CHIP. Thus, this hypothesis is an unlikely explanation of the fluorescence data.

These results then led to calcium uptake studies employing a lipid-permeable fluorescent dye to study intracellular bound calcium levels. Such experiments showed a marked increase in the intracellular bound calcium produced by all the platinum drugs, with CHIP and CBDCA having the greatest effect. Oxanthrazole, adriamycin and verapamil produced minimal effects, but all drugs decreased mitogen-stimulated calcium uptake into the cells, with *cis*-DDP having the least effect. The effects on mitogen-stimulated calcium uptake may be a reflection of these anti-tumor agents blocking mitogenesis. The marked increase in Ca^{2+} uptake into the cell produced by CHIP and CBDCA is interesting, since both drugs effect membrane lipids (in keeping with their chemical structures with hydrophobic side groups) and exert the greatest effects on calcium uptake. However, *cis*-DDP, which appears to affect the membrane by a charge–charge interaction, has a lesser effect on calcium uptake suggesting that the lipophylic site (site 2) may be more important in regulating calcium uptake in thymocytes than site 1 – the channel primarily affected by Tb^{3+}.

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