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Dipyridamole increases the cytotoxicity of cisplatin in human larynx cancer cells *in vitro*

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Abstract

This paper describes the effect of dipyridamole (DIP) on the cytotoxicity of cisplatin in HEp-2 human larynx cancer cells *in vitro* and the nature of the interaction between cisplatin and dipyridamole. Cytotoxic assays were performed to obtain the IC_{50} for cisplatin. The cells were treated with 0, 20, 40, 80, 120 or 200 µM cisplatin, with or without a single concentration of DIP and incubated for 60 min at 37ºC and 5% CO_2 for 3 days and then counted with a hemocytometer. The accumulation of cisplatin in the cells was measured by atomic absorption and fluorescence was used to determine the membrane binding constant of DIP. In the presence of 10, 20 and 30 µM DIP, the IC_{50} of cisplatin was reduced by 25, 60 and 82% in HEp-2 cells. Combination index analysis revealed that cisplatin and DIP interact synergistically. In larynx cancer cells, the accumulation of cisplatin increased by 13, 27 and 65% as the DIP concentration was increased from 10 to 20 and 30 µM, respectively. The binding constant of DIP to the cell membrane was estimated to be $(0.36 \pm 0.12\ mg/ml)^{-1}$ (N = 2) by fluorescence and cisplatin did not suppress DIP fluorescence. These results suggest that DIP significantly enhances cisplatin cytotoxicity in HEp-2 cells by increasing cisplatin accumulation, probably by altering the cell membrane as suggested by its binding constant. The results obtained reinforce the importance of combination therapy to reduce the doses of chemotherapeutic drugs and therefore the side effects of chemotherapy.

Key words
- Cisplatin
- Dipyridamole
- Synergism
- Tumor cells
- Cytotoxicity
- IC_{50}

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Introduction

Cisplatin has been used to treat different kinds of cancer although toxic side effects are known (1). The antitumor activity of cisplatin involves induction of intra- and interstrand cross-links that severely distort the DNA helix and block replication (1-3). Since cisplatin accumulation is a major determinant of its antitumor activity, modulators of cisplatin accumulation have received much attention.

Dipyridamole (DIP) has been used clinically in coronary heart disease for its antiplatelet and vasodilating activities (4,5) and is best known as an inhibitor of membrane nucleoside transport (6). DIP increases the cytotoxicity of several anti-cancer drugs in-

cluding 5-fluorouracil (7), methotrexate (8,9), adriamycin (10), etoposide (11), doxorubicin and vinblastine (12), and cisplatin (13). Jekunen et al. (13) showed that DIP synergistically enhanced the cytotoxicity of cisplatin in cisplatin-sensitive 2008 human ovarian carcinoma cells by a factor of 4.7, and in the cisplatin-resistant 2008/C13*5.25 subline by a factor of 5.8. In a nude mouse model with human bladder cancer (14), tumor size decreased by 20% when cisplatin was combined with DIP. Using human testicular carcinoma in the same model, complete tumor regression was achieved (14). Barberi-Heyob et al. (15) found that DIP synergistically increased the growth-inhibitory activity of cisplatin in MCF-7 human breast cancer cells. Perussi et al. (16) have studied the potentiation of cisplatin cytotoxicity by DIP in two human breast cancer cells, one of them cisplatin sensitive (MDA/S) and the other cisplatin resistant (MDA/R). In the presence of 30 µM DIP, the IC_{50} of cisplatin was reduced by 39% for both cell lines. In the MDA/S cells, the cellular accumulation of cisplatin increased by 57 ± 8% in the presence of 30 µM DIP, which did not affect the accumulation of cisplatin in MDA/R. The cited investigators suggested that the enhancement of cisplatin cytotoxicity by DIP in MDA/S cells may be related to a DIP-induced increase in cisplatin accumulation, but the enhanced cytotoxicity in MDA/R cells employs a mechanism that does not involve an increase in the cellular accumulation of cisplatin.

In the present study we report the enhancement of cisplatin cytotoxicity by DIP in human larynx cancer cells (HEp-2) that is probably related to a DIP-induced increase in cisplatin accumulation.

Material and Methods

Chemicals

Cisplatin, cis-diaminedichloroplatinum (II), and DIP, 2,6-bis (diethanolamine)-4,8-dipiperidinopyrimido [5,4-d] pyrimidine were purchased from Sigma (St. Louis, MO, USA). Cisplatin stock solutions were made fresh 24 h before each experiment in unsupplemented medium to minimize the hydrolysis of cisplatin. Dipyridamole stock solutions were prepared in DMSO and kept in the dark at 4ºC. The maximum DMSO concentration used was 0.1%. Drug solutions were sterilized via syringe filtration just before use. All chemicals were of analytical quality and were used as purchased.

Cell culture

HEp-2, a human larynx cancer cell line obtained from Adolfo Lutz Institute, São Paulo, SP, Brazil, was grown as a monolayer in 25-cm^2 flasks with Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, streptomycin and ampicillin at 37ºC in a humidified atmosphere of 5% CO_2 in 95% air. Confluent cell cultures were harvested with Versene (a solution constituted mainly of 0.526 mM EDTA) and removed from the flask with phosphate-buffered saline (PBS) solution. Cells were centrifuged, resuspended in medium and counted with a hemocytometer. Cell viability was assessed by the Trypan blue exclusion method.

Cytotoxic assays

HEp-2 cells were seeded on Petri dishes at 4.10^4 cells/dish and treated with 0, 20, 40, 80, 120 or 200 µM cisplatin in unsupplemented medium, with or without a fixed concentration of DIP ranging from 0 to 35 µM. It should be mentioned here that DIP is insoluble in aqueous solutions at concentrations above 40 µM. The cells were incubated with the drug(s) for 60 min at 37ºC and 5% CO_2. After drug removal ordinary supplemented medium was added and the cells were incubated for 3 days. The cells were

harvested with Trypsin, fixed in 37% formaldehyde and counted using a hemocytometer. All experiments were carried out in duplicate. The number of cells for 0.0 µM cisplatin and 0.0 µM DIP was taken as the control, corresponding to 100% cell survival. An index of survival was calculated at each cisplatin concentration, i.e., the number of live cells with the drug divided by the number of control live cells without the drug. Median effect analysis was used to determine the mean inhibitory concentration (IC_{50}) for cisplatin and for DIP (17). Data were visualized graphically by plotting the index of cell survival against the cisplatin concentration. The degree of synergism between cisplatin and DIP was determined by using combination index analysis at a non-constant ratio as described above. According to Chou and Talalay (18), combination index <1 stands for synergism while combination index = 1 indicates summation and combination index >1 suggests antagonism (18).

Data are reported as means ± SD. The Student unpaired *t*-test was used to determine differences between pairs of means, with the level of significance set at $P < 0.05$.

Determination of cisplatin accumulation in the presence of dipyridamole

The method used to calculate the accumulation of cisplatin has been described (19). Briefly, cells were seeded into 60-mm plastic culture dishes and grown to confluence as monolayers in ordinary supplemented medium at 37ºC and 5% CO_2. The cells were incubated with 200 µM cisplatin in the presence of 0, 5, 10, 20 or 30 µM DIP in unsupplemented medium for 60 min at 37ºC, then washed four times with ice-cold PBS. Next, 1.0 ml of 0.1% Triton X-100 in 0.1 N HCl was added to each dish and the cells were scraped from the bottom surface. The detached cells were frozen in cryogenic vials for at least 24 h. Upon thawing, the cells were sonicated at 7 watts for 15 s. Twenty-microliter aliquots of the lysate were used for platinum analysis with a Perkin-Elmer Zeeman Atomic Absorption Spectrometer Model 4110ZL (Wellesley, MA, USA). Cisplatin accumulation is reported as pmol of platinum/mg protein. The protein content of each sample was measured by the method of Lowry et al. (20). One-way analysis of variance (ANOVA) was used to analyze the atomic absorption data.

Determination of the association constant of dipyridamole with the cell membrane

The determination of the association constant of DIP with the cell membrane was performed by titration. The cells in a high-density cell suspension were lysed by sonication or by adding a hypotonic solution and centrifuged. The protein concentration of the supernatant and membrane fraction was determined by the method of Lowry et al. (20). DIP solution (1 µM) was added to variable concentrations of membrane suspensions (0 to 1.30 mg of protein/ml), the samples were mixed in a Vortex mixer and incubated for 60 min at 37ºC and 5% CO_2. The samples were then centrifuged at 4000 *g* for 10 min and the fluorescence spectra of the supernatant were obtained. The pellet obtained by centrifugation was washed twice with 1% phosphate buffer and solubilized in 1 ml of 2% Triton X-100 and 10% NaCl. The supernatant contains the cytoplasmic constituents and the pellet contains the mitochondria as well as nuclear and cell membranes. The fluorescence of the solubilized pellet and the supernatant was detected with a Hitachi F-4500 fluorometer (Ibaraki, Japan) in the region of 400-600 nm with excitation at 415 nm since DIP absorbs at this wavelength. A 1-µM DIP solution was used as a standard for fluorescence intensity. Data from the titration were analyzed by directly fitting to the law of mass action (21,22) or as double reciprocal plots of fluorescence intensity changes (ΔF) and membrane concentrations.

Considering the equilibrium:

$$D + M \Leftrightarrow D - M \qquad \text{(Eq. 1)}$$

where D represents the drug and M the cell membrane, the total fluorescence observed is due to the free and bound species of D. ΔF represents the difference between the initial fluorescence intensity (F_0) in the absence of the cell membrane and the fluorescence intensity of the drug in the presence of cell membrane (F). This difference (ΔF) is related to the quantity of the drug associated with the membrane. In order to quantitate the association constant for the binding of DIP to the membrane (K_b) and ΔF_{max}, the fluorescence data for the supernatant obtained by titration of the drug with membrane suspensions were treated by the double reciprocal method (23,24). This treatment is based on the following equation:

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \frac{1}{\Delta F_{max}} \times \frac{1}{K_b} \times \frac{1}{[M]} \qquad \text{(Eq. 2)}$$

where ΔF_{max} is the variation of fluorescence intensity between the fluorescence intensity in the absence of the membrane and the fluorescence emission intensity of the drug at a saturation membrane concentration (based on the membrane protein concentration). This value can be obtained from the fit of the experimental data using Equation 2.

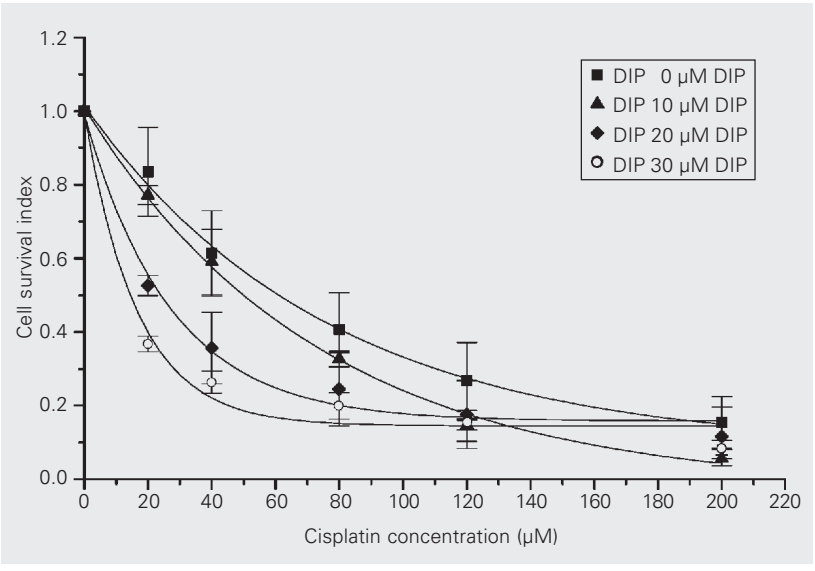

Figure 1. Survival index of HEp-2 cells as a function of cisplatin concentration in combination with dipyridamole (DIP). Cells were treated with several cisplatin concentrations (0, 20, 40, 80, 120 and 200 μM) combined with fixed DIP concentrations (0, 10, 20 and 30 μM) for 1 h at 37ºC in the presence of 5% CO_2. Data are reported as means ± SD for 5-13 replicates.

Investigation of complex formation between dipyridamole and cisplatin

The possibility of a direct interaction between DIP and cisplatin was investigated by determining the suppression of the fluorescence of 5 μM DIP after each addition of 20 μl of a 2 mM cisplatin solution prepared in phosphate buffer, pH 7.2. The fluorescence spectrum of each sample was obtained from 425 to 600 nm with a Hitachi F-4500 fluorometer, with excitation at 415 nm. Sample absorbance was monitored after each addition of the titrating compound and the spectra from 250 to 600 nm were recorded with a Shimadzu UV-1601 PC spectrophotometer.

Results and Discussion

Figure 1 shows the indices of cell survival for HEp-2 cells as a function of cisplatin concentration, in the absence and presence of 10, 20 and 30 μM DIP. At each cisplatin concentration, the indices of survival of the cells were reduced in the presence of DIP. In this cell line, the survival curve for cisplatin alone was significantly different from that for cisplatin plus DIP ($P < 0.001$). These results suggest that the cytotoxicity of cisplatin is increased in the presence of DIP. Table 1 presents the IC_{50} values obtained from plots shown in Figure 1. In HEp-2 cells, the IC_{50} of cisplatin was reduced by 25, 60 and 82% in the presence of 10, 20 and 30 μM DIP, respectively. The degree of enhancement of cisplatin cytotoxicity increased with DIP concentration. Perussi et al. (16) observed that the IC_{50} value for cisplatin in human breast cancer cells sensitive to cisplatin (MDA/S) de-

creased by 39% in the presence of 30 µM DIP, suggesting that HEp-2 cells are more sensitive to the combination of the drugs.

Figure 2 shows the sensitivity of the cells to DIP alone. DIP alone decreased the survival of HEp-2 cells by 10, 17 and 29% at concentrations of 10, 20 and 30 µM, respectively. This reduction was statistically significant ($P < 0.01$) for 20 and 30 µM DIP. The IC_{50} of DIP was estimated to be 94 ± 10 µM since the solubility of DIP is very low in aqueous solutions and just a short range of DIP concentrations could be used in the assay (0 to 35 µM DIP). The cytotoxic effect of cisplatin was 1.6 times more potent than that of DIP alone in these cells (IC_{50} was 94 µM for DIP and 60 µM for cisplatin).

Table 2 presents the affected fractions and the combination index obtained for the cytotoxic experiments performed at a non-constant ratio with cisplatin in combination with DIP in HEp-2 cells. Combination index analysis indicated that this parameter was lower than one in 93% of the combinations used, suggesting that the interaction between cisplatin and DIP is synergistic (18). Similar results of synergistic interaction were reported by Jekunen et al. (13) for an ovarian carcinoma cell line treated with the combination of these two drugs.

The data in Figure 1 suggest that the fraction affected by 80 µM cisplatin with 10 µM DIP was similar to the fraction affected by 40 µM cisplatin with 20 µM DIP and 20 µM cisplatin with 30 µM DIP. However, the indices obtained for these drug combinations were different (Table 2), suggesting that the interaction of cisplatin with DIP was synergistic when low cisplatin concentrations were combined with moderate and high DIP concentrations, and moderately synergistic when low cisplatin concentrations were combined with low DIP concentrations. Furthermore, Figure 1 indicates that the fraction affected by 120 µM cisplatin with 10 µM DIP may be similar to the fractions affected by 120 µM cisplatin with 20 µM DIP and by 120 µM cisplatin with 30 µM DIP. The indices obtained for these drug combinations (Table 2) were about the same, suggesting that the interaction of cisplatin with DIP was also synergistic at high cisplatin concentrations.

Table 1. Effect of dipyridamole on the cytotoxicity of cisplatin in the human larynx HEp-2 cell line.

Dipyridamole (µM)	IC_{50} (µM)	Decrease in the IC_{50} (%)
0	60 ± 9	–
10	45 ± 2	25
20	24 ± 2	60
30	11 ± 3	82

The cells were exposed to several cisplatin concentrations (0, 20, 40, 80, 120 and 200 µM) with and without dipyridamole for 1 h at 37ºC (N = 5-13). Data are reported as means ± SD. The IC_{50} values for the association of the drugs were statistically different ($P < 0.05$; ANOVA).

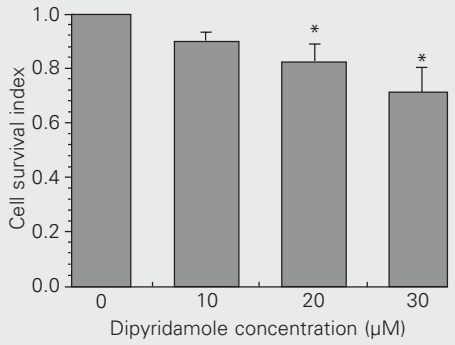

Figure 2. Effect of dipyridamole (DIP) on the survival index of HEp-2 cells. Cells were treated with several DIP concentrations (0, 5, 10, 15, 20 and 30 µM) for 1 h at 37ºC in the presence of 5% CO_2. Each column represents the mean ± SD for two experiments each performed in duplicate. *$P < 0.005$ compared to no DIP (ANOVA).

Table 2. Combination indexes for cisplatin and dipyridamole (DIP) in the HEp-2 cell line obtained by the median effect analysis.

Cisplatin (µM)	DIP concentration (µM)					
	10		20		30	
	f_a	CI	f_a	CI	f_a	CI
20	0.23	1.17	0.47	0.64	0.62	0.46
40	0.49	0.83	0.64	0.60	0.74	0.46
80	0.66	0.85	0.75	0.73	0.80	0.58
120	0.84	0.66	0.82	0.75	0.86	0.66
200	0.96	0.55	0.88	0.92	0.92	0.75

Data are reported as means ± SD for 5-13 replicates. CI = combination index; f_a = affected fraction.

In order to assess the mechanism involved in the synergism between cisplatin and DIP, cellular accumulation of cisplatin was determined in the presence of DIP. Table 3 shows that at 0.0 µM DIP the accumulation of cisplatin in HEp-2 cells was 830 ± 400 pmol Pt/mg membrane protein and this value increased with increasing DIP concentrations from 0 to 30 µM. In the presence of 30 µM DIP, the cisplatin accumulation increased significantly by 65% to 1373 ± 400 pmol Pt/mg protein ($P < 0.005$), while the IC_{50} value for cisplatin combined with 30 µM DIP decreased by 82%. These results suggest that the increase in cisplatin accumulation by DIP increased the cytotoxicity of cisplatin in HEp-2 cells.

Table 3. Effect of dipyridamole (DIP) on the accumulation of cisplatin in human larynx HEp-2 tumor cells.

Dipyridamole (µM)	pmol Pt/mg protein	Increase in Pt (%)
0	830 ± 400	0
10	934 ± 470	13.0
20	1053 ± 500	27.0
30	1373 ± 401	65.0

All cells were treated with 200 µM cisplatin for 1 h at 37ºC in combination with DIP (0, 10, 20 and 30 µM). Data are reported as means ± SD for 5 replicates. Platinum (Pt) content for the association of 30 µM DIP is statistically different from that of cisplatin itself ($P < 0.005$; ANOVA).

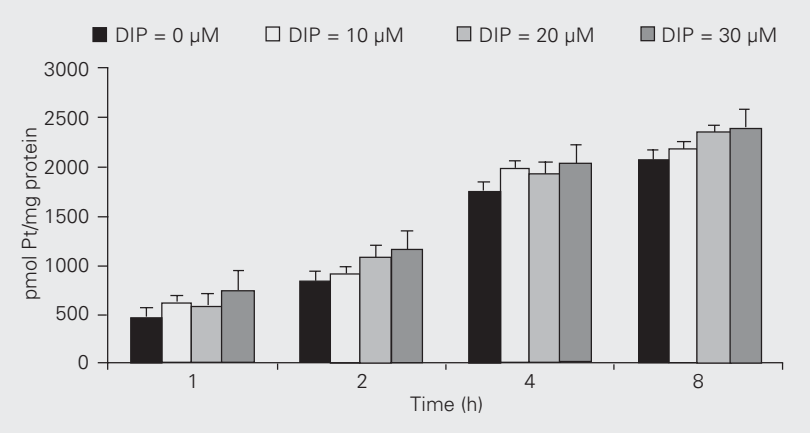

Figure 3. Effect of dipyridamole (DIP) and incubation time on intracellular accumulation of cisplatin in the human larynx tumor cell line HEp-2 at 37ºC and 5% CO_2. Data are reported as means ± SD for 5 replicates. Platinum (Pt) content for each incubation time was statistically different ($P < 0.005$) from that of 1 h of incubation.

Experiments were performed to investigate the effect of incubation time on the intracellular accumulation of cisplatin in HEp-2 cells. When cisplatin was combined with DIP (0, 10, 20 and 30 µM) (Figure 3) the increase in incubation time of the drugs was found to lead to an increase in intracellular cisplatin concentration. For each incubation time used there was a significant increase ($P < 0.005$, ANOVA) in platinum accumulation. So, we may conclude from these results that the amount of intracellular cisplatin depends on DIP concentration as well as on the duration of incubation with the drugs.

Titrations of membrane suspensions have indicated that the association depends on the increase of the membrane concentration in solution. The results were closely similar to those obtained for the interaction of DIP with mitochondrial and erythrocyte membranes (21,22). As the membrane concentration increased, the fluorescence intensity in the supernatant decreased, while it increased in the pellet. These alterations in the emission spectra were used to estimate the association constant for DIP with the cell membrane.

A plot of $1/\Delta F$ (data for the supernatant fluorescence which was proportional to free DIP concentration) as a function of 1/[M] may be used to obtain K_b, the binding constant. The results obtained for both treatments, the mass-action law and the method of double reciprocal plot, are shown in Figure 4. The K_b value obtained by the double reciprocal plot was 0.36 ± 0.12 (mg protein/ml)$^{-1}$. This value was obtained as the ratio of the intercept to the slope in the linear fit shown in the insert in Figure 4 and ΔF_{max} was obtained from the reciprocal of the intercept value. This value for the binding constant was quite similar to those obtained for the association constant of DIP with the erythro-

cyte ghost membranes of 0.40 ± 0.02 (mg protein/ml)$^{-1}$ (22) and for the association constant of DIP with the mitochondrial membrane of 0.8 ± 0.1 (mg protein/ml)$^{-1}$ (21). On the basis of the dependence of the DIP fraction in the pellet on membrane concentration, we found that 36% of the drug was present in the pellet. This means that with 1 µM DIP concentration and an excess of membrane, about 36% of the drug was bound. This value is quite close to the maximum DIP saturation of 47% reported for the mitochondrial membrane (21).

Borges et al. (25) studied the interaction of DIP with bovine serum albumin (BSA) and membrane model systems (micelles). It was shown that DIP binds strongly to BSA, in agreement with the high level of DIP binding to human plasma albumin. This study (25) also showed that DIP binds more strongly to neutral micelles and the results of fluorescence suppression suggest that DIP is located in the interface of the micelle, close to the beginning of the hydrophobic region. In fact, DIP incorporation seemed to occur in a region close to the border of the hydrophobic and polar parts of a phospholipid monolayer (26). It has been shown that the protective effect of DIP against the lipid peroxidation caused by cumene hydroperoxide in mitochondrial membrane was strongly dependent on the duration of incubation with the drug prior to the addition of the oxidant (27). A similar feature was observed for the protective effect of DIP on red blood cell lysis. The protection was quite sensitive to the time of incubation with the drug and to its concentration (22).

The experiments of fluorescence suppression were performed in order to determine the complex formation between DIP and cisplatin. The fluorescence at 480 nm of a 10 µM DIP solution in 0.02 M phosphate buffer, pH 7.2, was monitored after each addition of cisplatin (0 to 0.4 mM final cisplatin concentration). The Stern-Volmer plot (data not shown) resulted in a straight line parallel to the x-axis in the entire range of the suppressor concentration used. Thus, the Stern-Volmer constant (K_{SV}) could not be obtained in this case because there was no fluorescence suppression, indicating that no complexation occurred between DIP and cisplatin at the concentrations used.

The present study has demonstrated that the combination of cisplatin and DIP leads to an increased cytotoxicity in HEp-2 cells. A reduction of up to 82% in the IC_{50} of cisplatin was obtained when cisplatin was combined with 30 µM DIP. This reduction was much greater than that observed in other cell lines, such as in cisplatin-sensitive and cisplatin-resistant breast tumor cell lines previously studied by us (16). In that investigation, in the presence of 30 µM DIP, the IC_{50} of cisplatin was reduced just by 39% for both cell lines. This result suggests that the combination of these drugs can also improve the cytotoxicity of cisplatin in resistant cell lines. The results of that study as well as others in the literature (13) permit us to conclude that the co-administration of cisplatin and DIP is feasible and may permit the treatment of

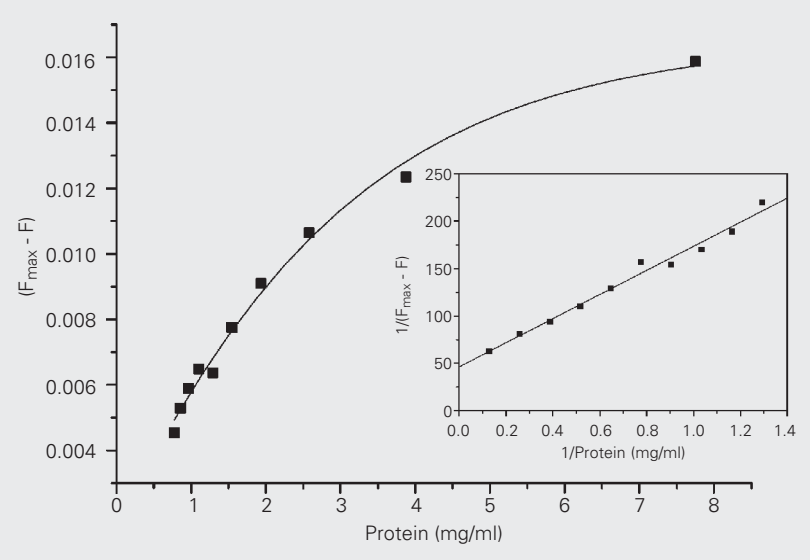

Figure 4. Analysis of the titration of dipyridamole (DIP) with HEp-2 cell membrane by fluorescence for the calculation of the association constant. Inset: Double reciprocal plot of the change in DIP fluorescence emission *vs* 1/protein. DIP concentration of 1 µM, excitation at 415 nm and emission at 495 nm.

cancer patients with cisplatin concentrations that are effective in killing cisplatin-resistant cancer cells by reducing or eliminating the severe side effects of high drug concentrations. In this way, resistance to cisplatin may be overcome.

The atomic absorption experiments showed that DIP increases the uptake of cisplatin by cells in a concentration-dependent manner. These results agree with those reported by Perussi et al. (16) and by Jekunen et al. (13) who observed an increased accumulation of cisplatin due to DIP, but without increasing Trypan blue or propidium iodide uptake or changing cell size. The cited investigators concluded that the DIP-induced increase in cisplatin accumulation was not associated with a nonspecific increase in membrane permeability. In the present study we showed that the cellular accumulation of cisplatin is concentration and time dependent. It has been shown that DIP incorporation into model membranes is time dependent (27,22).

The analysis of the median effect showed that the interaction between these two drugs was synergistic and our fluorescence suppression experiments showed no complexation between DIP and cisplatin. Fluorescence experiments also allowed to determine the binding constant of DIP to the cell membrane as 0.36 ± 0.12 (mg protein/ml)$^{-1}$, a value similar to those obtained for the binding of this drug to mitochondrial and red blood cell membranes.

Our results suggest that the enhancement of cisplatin cytotoxicity by DIP in HEp-2 cells may be related to a DIP-induced increase in cisplatin accumulation. The results obtained support the importance of combined therapy to reduce the doses of chemotherapeutic drugs and therefore the side effects of chemotherapy.

Acknowledgments

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