Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 1115–1120

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

COMPLEXATION STUDY AND ANTICELLULAR ACTIVITY ENHANCEMENT BY DOXORUBICIN-CYCLODEXTRIN COMPLEXES ON A MULTIDRUG-RESISTANT ADENOCARCINOMA CELL LINE

Anouar Al-Omar[1], Souad Abdou[1], Laurence De Robertis[2], Alain Marsura[1]*, Chantal Finance[1]

[1] *Unité Mixte de Recherche CNRS -Université, Structure et Réactivité des Systèmes Moléculaires Complexes, Université Henri-Poincaré, Nancy-1; Faculté des Sciences Pharmaceutiques et Biologiques 5, rue A. Lebrun, BP 403, F-54001 Nancy Cedex.*

[2] *Centre D'Etudes et de Recherches sur les Macromolécules Végétales, U.P.R. CNRS 5301, B.P. 53, F-38041 Grenoble Cedex 9.*

Received 4 November 1998; accepted 10 March 1999

Abstract : Ability of molecular complexes of [Doxorubicin (DX)-cyclodextrin (Cd)] to enhance the anticellular activity of antineoplasic drug Doxorubicin and to reverse its multidrug resistance has been investigated. A spectroscopic study of the α,β, and γ-[DX-Cds] complexes has been investigated in relation to their biological effects on a multidrug resistant (MDR) human rectal adenocarcinoma cell line (HRT-18). A ten fold enhancement of DX anticellular activity in presence of β-cyclodextrin alone was detected.

In present day cancer therapy, Doxorubicin (Fig. 1) and Daunorubicin are the most frequently used members of the anthracycline group. The main structure of these cytostatic drugs is constituted of a tetracyclic anthraquinoid aglycone linked to an oligosaccharide moiety as e.g. the daunosamine amino-sugar.[1]

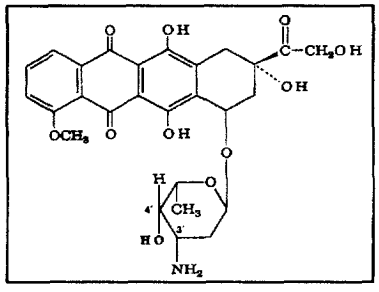

Fig. 1 Chemical Structure of Doxorubicin

However, the cardiotoxicity and the occurring MDR cell lines actually remain the major clinical problems.[2,3] In order to improve the therapeutic efficiency of antineoplasic anthracyclines, targetting approaches have been recently explored, using liposome,[4-7] antibody[8,9] or immunoliposome[10] strategies. Elsewhere, it has now been well established that Cds could be employed to obtain inclusion complexes which have shown to exhibit significant therapeutic index improvements and which are now employed in

* E Mail : marsura@srsmc.u-nancy.fr ; Fax : (33) 03.83.17.88.63

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00150-X

pharmaceuticals [11]. The present work treats the cyclodextrins as carriers to enhance the anticellular activity and to reverse the MDR of Doxorubicin drug on a human rectal adenocarcinoma cell line, HRT-18.

An attempt was made to complex Doxorubicin [12] with α, β, γ-Cds in water solutions [13] and the complexation features were followed by spectrofluorimetry and circular dichroism.[14] The emission spectra of Doxorubicin (Fig. 2) show almost 50% decrease in fluorescence intensity at 600 nm in presence of increasing amounts of β and γ-Cds.

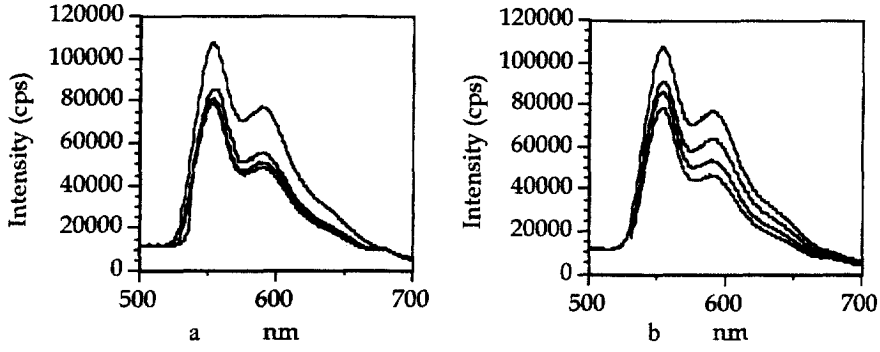

Fig. 2 Fluorescence emission spectra of DX : a) in presence of increasing amounts of β-Cd; b) in presence of increasing amounts of γ-Cd

The effects of [DX-Cds] complexes have been studied on HRT-18 Human rectal adenocarcinoma cells [15] along the native α, β, γ-Cds taken as references. The latter do not exhibit any inhibition of the cellular viability at concentrations between 1 to 4 mM. On the contrary an increasing viability with β-Cd which stimulates the dehydrogenase cell activity between 1 and 3 mM concentrations was observed (Fig. 3a).

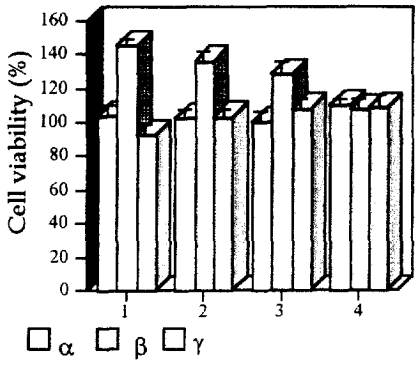

Fig. 3a. Anticellular activity of unmodified Cds. 1= 1mM; 2= 2mM; 3= 3mM; 4= 4mM.

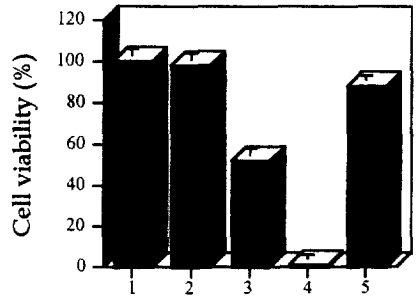

Fig. 3b. Anticellular activity of DX and [DX-Cds] on MDR adenocarcinoma cells : 1= untreated cells, 2= DX (1.7. 10^{-4} M); 3=[DX-α-Cd]; 4=[DX-β-Cd]; 5=[DX-γ-Cd]. The concentration of each Cd in [DX-Cds] was 2.2 10^{-3} M.

The anticellular activity of the DX and [DX-Cds] complexes on HRT-18 cells has been evaluated on cells which have been exposed to pure DX and [DX-Cds] complexes during three days.

After this time the anticellular activities of each were compared. As shown in Fig.3b one can notice that free DX alone exhibits a very low toxicity. On the cells treated with 1.7 10^{-4} M of DX no significant decrease in cell viability is registered. In comparison, more than 95 % of inhibition is observed in the case of the [DX-β-Cd] for the same concentration of DX, whereas the cell mitochondrial enzymatic activity was poorly affected (40%) with [DX-α-Cd] and appears to be unaffected (less than 10%) in the case of [DX-γ-Cd]. Furthermore, the IC_{50} was determined in each case (data not shown) and revealed a 10-fold enhancement of [DX-β-Cd] cytotoxicity (IC_{50} = 1.3 10^{-4} M, compared to those of the [DX-γ-Cd] which was close to 1.3 10^{-3} M). A Cd /dose-response study of DX cellular toxicity has shown (unpublished data) that the cytotoxic effect of DX was independent of the β-Cd concentration. Consequently, anticellular activity enhancement of DX is not due to an induced metabolic effect of the cyclodextrin alone on the cell. Concurrently, the intracellular uptake concentration of DX function of inoculation time was evaluated in conditions described below.[16] The observed values (Table 1) show that the complexation does not allow an increase in the amount of DX put into the cells over a 6h to 72h exposure time.

Time of exposure (hrs)	Intracellular conc. of DX (free DX; IF in cps)	Intracellular conc. of DX ([DX-β-Cd]; IF in cps)
6	$1.8.10^6$	$1.4.10^6$
12	$2.0.10^6$	$2.0.10^6$
24	$2.5.10^6$	$2.5.10^6$
48	$2.7.10^6$	$2.8.10^6$
72	$3.1.10^6$	$3.3.10^6$

Table 1. Intracellular uptake of DX function of time[16] *expressed in fluorescence intensities (cps).* $\lambda_{ex.}$ *= 467 nm;* λ_{em} *= 553 nm. Standard deviation = 10%*

Considering the above results, some features should be discussed in relation to the experimental spectroscopic data. ^{1}H-NMR spectra (not represented here), recorded both on free DX and in presence of α, β and γ-Cds increasing concentrations, show as previously reported[17] the DX anthraquinone moiety inclusion by the larger γ-Cd cavity. This inclusion was unrecovered with β-Cd or α-Cd related to the inadequate cavity sizes. Furthermore, and unfortunately, we were unable by ^{1}H-NMR to confirm a suspected molecular association between the keto-carbocyclic moiety of DX and β-CD because of almost complete overlapping of the involved DX protons and those of the cyclodextrin (400MHz 1D NMR spectra).

To circumvent this problem, and being better than NMR, circular dichroism was used to approach possible molecular conformational changes and interactions of the ketonic carbocyclic and chiral moiety of DX with the Cds hosts. Referring to Beckers work,[18] the corresponding CD spectra of DX and [DX-Cds] have been recorded (Fig. 4). As previously mentioned, significantly increased absorption was observed on the anthraquinone chromophore (495 nm) in presence of γ-Cd (Fig 4. *iii*) and confirms the anthraquinone inclusion.

This effect was naturally not observed for α- and β-Cds due to their too small internal cavity sizes (6-7 Å) that could not allow inclusion. Nevertheless, CD spectra possible changes, induced by complexation in the 300 to 400 nm region, have never been analysed. As shown above in Fig. 4.(*ii, iii*) an increasing absorption along peak maximum inversion occurs in the case of β- and γ-Cd but not in the case of α-Cd. These results suggest that an interaction, different from the classical inclusion, occurs between the DX and β- or γ-Cd.

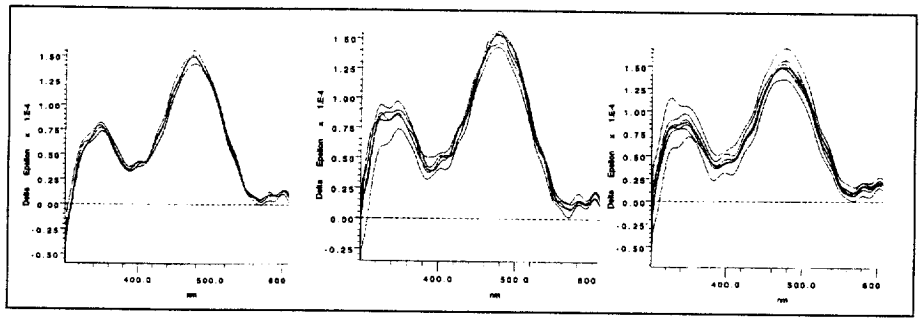

Fig 4. CD spectra of i : [DX-α-Cd], ii: [DX-β-Cd] and iii: [DX-γ-Cd] complexes.

Summarising all the above results, inclusion of the DX anthraquinone moiety in γ-Cd has been demonstrated by several methods. Despite this preferential inclusion one should observe that the DX anticellular effect remains unmodified. This case is compatible with a free drug action, since the [DX-γ-Cd] possesses the same activity level.

On the contrary, it is clear that anthraquinone does not participate in an inclusion complex with β-Cd. Nevertheless, the anticellular activity of DX in that case is highly enhanced whereas cell viability measurements show the Cds are non cytotoxic. Considering and excluding any anticellular activity of the cyclodextrin itself, the tenfold activity enhancement and medium effects obtained respectively with the [DX-β-Cd] and [DX-α-Cd] in which the anthraquinone is free of inclusion become more controversial. A possible protective effect of β-Cd against the enzymatic metabolisation of the amino-sugar moiety, that leads to the drug inactivation, is not sufficient to explain the results obtained with α- and γ-Cd. A possible use of β-cyclodextrin itself *in vivo* after IV administration, will be limited by the β-Cd concentration level which exhibits a renal toxicity by precipitation at high concentration. Nevertheless, this limitation does not exist in case of α-Cd or with the soluble hydroxypropyl-β-Cd (HPBCd) analog.

Today, we believe that any new proposals to explain the surprising modification of the anticellular activity of DX and MDR reverse effect in presence of β-Cd remain hazardous without supplementary data on the localisation of the Cds . In a future work the use of labelled Cds will enable us to follow what will happens to the ligand within the cell.

Acknowledgements : MRES, the région Lorraine, the Fondation pour la Recherche Médicale are acknowledged for their financial support and Pharmacia S.A. laboratory for kindly providing Doxorubicin samples, Wacker Chemie S.A. for their generous gift of cyclodextrins. We are indebted to Mrs Nicole Marshall for correcting the manuscript.

References and Notes

1 Booser, D.J.; Hortobaygi, G.N. *Drugs* **1994,** *47*, 223.

2 Singal, P.K.; Pierce, G.N. *Amer. J. Physiol.* **1986**, *250*, H419.

3 Pearson, C.; Cunningham, C. *TIBTECH* **1993**, *11*, 511.

4 Perez-Soler, R.; Priebe, W. *Cancer Res.* **1990**, *50*, 4260.

5 Forssen, E.A. *Adv. Drug Del. Rev.* **1997**, *24*, 133.

6 Oudard, S.; Theirry, A.; Jorgensen, T. J.; Rahman, A. *Cancer Chemother. Pharmacol.* **1991**, *29*, 259.

7 Warren, L.; Jardillier, J.-C.; Malarska, A.; Akeli, M.G. *Cancer Res.* **1992**, *52*, 3241.

8 Mickisch, G.H.; Pai, L.H.; Gottesman, M.M.; Pastan, I. *Cancer Res.* **1992**, *52*, 4427.

9 Trail, P.A.; Wilner, D.; Lasch, S.J.; Henderson, A.J.; Hofstead, S. *Science* **1993**, 261, 212.

10 Emanuel, E.; Kedar, E.; Bolotin, E.M.; Smorodinsly, N.I., Barenholz, Y. *Pharm. Res.* **1996**, *13*, 861.

11 Li, S.; Purdy, W.C. *Chem. Rev.* **1992**, *92*, 1457.

12 The Doxorubicin was purchased from Pharmacia (Saclay, France); and the cyclodextrins from Wacker-Chemie GmBH (Lyon, France). The products were solubilised in distilled water and sterilised by filtration through an 0.22 μm membrane (Millipore, Molsheim, France) before use. The human rectal adenocarcinoma cell line HRT-18 was supplied by Laporte (INRA,Jouy-en-Josas, France) and was grown in RPMI 1640 medium (ICN Biomedicals Inc. Costa Mesa, CA, USA) supplemented with fetal calf serum (Life Biotechnologies, Eragny, France) and Ofloxacin (Roussel Uclaf, Romainville, France). The cells were passaged using Trypsin-EDTA (Difco, Michigan, USA). The cellular viability was evaluated with MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma, St-Louis, MO, USA).

13 Doxorubicin-Cds complexes were prepared taking in account the solubility data according to Higuchi-Connors.[19] The great tendency of anthracyclines to absorb on glass in neutral solutions and to cause photochemical degradations, involves to silanising all the vials with dimethyl chlorosilane and operating in the dark. A doxorubicin water solution ($1.7.10^{-4}$ M; 2mL) was gradually added to varying concentrations of α, β or γ-Cd solutions. The final solutions (10 mL) were equilibrated overnight under stirring in the dark at r.t.. Finally, solutions were lyophilised and the resulting powders were stored under argon and in the dark at -20°C.

14 Fluorescence spectra have been recorded on a Fluorolog II (Spex-Jobin-Yvon) photon counting spectrometer, CD spectra on a CD6 (Jobin Yvon) spectrometer.

15 The anticellular assays on adenocarcinoma cells were done over HRT-18 cells maintained as a monolayer culture in RPMI 1640 with 2 mmol L-glutamine supplemented with 20% (v/v) heat inactivated (56°C, 30 min) fetal calf serum and 10mg/mL ofloxacin at 37°C in a 95 : 5 air/CO_2 gaz mixture. The cells were passaged twice weekly by enzymatic disaggregation using trypsin -EDTA and seeded at 3 to $3.5.10^5$ cells / mL in fresh medium. Confluent HRT-18 cells, grown in 96-well flat bottom culture plates at 4.10^4 cells per well were incubated with Doxorubicin or Doxorubicin/Cds complexes for 72 h at 37°C. After this period, the drug cytotoxic effect was evaluated by the determination of the mitochondrial dehydrogenase activity using MTT (3-[4-5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay as previously described.[20] Absorbances in viability assays were measured on a Titertek Multiscan MCC/340 MKII

(Labsystems, Helsinki, Finland) at 540 nm on cell culture 96 well microplates. Results shown are average values from triplicate experiments.

16 The intracellular Doxorubicin uptake was evaluated on confluent cells grown in 6 well microculture plates inoculated with DX ($1.7\ 10^{-4}$ M) or [DX-β-Cd]; (DX = $1.7\ 10^{-4}$ M; Cd= $2.2\ 10^{-3}$M).The plates were incubated at 37°C under CO_2 (5%) for different post-inoculation times without any change of medium. Each assay was tested in triplicate. Three blank wells received only the culture medium and three wells of cells without drugs serve as controls. After incubation, the medium was removed, the cells washed with saline phosphate buffer (PBS, pH = 7.3, 37°C) and PBS (300 μL) containing SDS (1%) was added for 15 min at 37°C. The cell suspension was harvested with cold PBS (3mL). The amount of DX was quantified by spectrofluorimetry. Each experiment was done in triplicate, with 10% standard deviation average.

17 Djedaïni, F.; Lechat, F.; Wousseidijewe, D.; Perly, B. *In Minutes,* Duchêne D., Ed.; Edition de Santé , Paris 1990.

18 Beckers, O.; Beijnen, J.H.; Otagiri, M.; Bult, A.; Underberg, W.J.M. *Pharm. Biomed. Anal.* **1990**, *8*, 671.

19 Higuchi, T.; Connors, K.A. *In Advances in Analytical Chemistry and Instrumentation*, Reilley, C.N., Ed. Interscience Publishers, John Wiley & Sons, 1965; Vol. 4, pp 117-212.

20 Attioui, F.; Al-Omar, A.; Leray, E.; Parrot-Lopez, H.; Finance, C.; Bonaly, R. *Biol. Cell.* **1994**, *82*, 161.