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ANTIVIRAL PROPERTIES OF PSORALEN DERIVATIVES: A BIOLOGICAL AND PHYSICO-CHEMICAL INVESTIGATION

G. PALÙ,*§ M. PALUMBO,† R. CUSINATO,* G. A. MELONI* and S. MARCIANI MAGNO‡

*Institute of Microbiology, University of Padova Medical School, Via A. Gabelli, 63-35100 Padova, Italy; †Biopolymer Research Centre, C.N.R., Institute of Organic Chemistry, University of Padova, Via Marzolo, 1-35100 Padova, Italy; and ‡Centro di Studio sulla Chimica del Farmaco e dei Composti Biologicamente Attivi, C.N.R., Institute of Pharmaceutical Chemistry, University of Padova, Via Marzolo, 5-35100 Padova, Italy

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Abstract—Two isomeric psoralen derivatives (I and II in Fig. 1) bearing charged side chains, have been tested for activity against Herpes Simplex Virus type 1 (HSV-1) in the absence of u.v. irradiation. Striking differences have been observed both in antiviral and cytotoxic activity for the examined compounds, I being appreciably more effective. Metabolic and biochemical studies, as well as physico-chemical measurements indicate DNA as the major target. The different biological behaviour can be fully explained in terms of a modified affinity of the drugs toward DNA. The molecular basis for these findings probably stems from slightly different intercalation geometries, as shown by chiroptical studies. Comparable binding affinities for viral and cellular DNA fully account for lack of selective toxicity found *in vivo*. The present approach is proposed as a tool for the investigation of structure–function relationships in drug models.

Psoralens are a group of photobiologically active compounds currently employed in the treatment of vitiligo, psoriasis and other skin diseases [1]. The molecular basis for their action consists of an intercalation into the DNA double helix, followed by a C_4 photocycloaddition to the pyrimidine bases of nucleic acid, to yield a covalently bound drug-polynucleotide complex [2]. However, little is known on the biological behaviour of these drugs in the absence of irradiation. In particular the antiviral activity in the dark has been only occasionally investigated as a marginal aspect of the photochemical inactivation [3]. In this connection it seems also noteworthy that an antiviral effect is exhibited by a family of parent compounds, namely the antibiotics Novobiocin and Coumermycin A_1 [4–7], sharing with psoralens the coumarin moiety in the planar aromatic portion of their molecule. These observations prompted us to investigate the antiviral properties in the absence of irradiation of psoralen congeners chosen among a number of available compounds bearing charged side chains. The latter feature provides high water solubility and stronger interaction with the biological environment.

In the present paper we report on the effects on the replication of HSV-1 of two structurally related psoralen derivatives, namely 5(ω-diethylaminopropyloxy)psoralen hydrochloride (I) and 8(ω-diethylaminopropyloxy)psoralen hydrochloride (II). In an attempt to elucidate structure–function relationships, DNA binding affinity and complex stereochemistry were also investigated.

§ To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemical compounds. Compounds I and II were synthesized, purified and tritium labelled as previously described [8]. ^{3}[H]methyl thymidine (specific activity 40–60 Ci/mmole) and ^{3}[H]amino acid mixture were purchased from Amersham (Bucks, U.K.). Calf thymus DNA (type I) was obtained from Sigma Chemical Co. (U.S.A.). All chemical reagents were AnalaR grade.

Cells and virus. Vero, HEp-2 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS) (Gibco), antibiotics and L-glutamine (complete medium). HSV-1 was a clinical isolate identified by its digestion pattern with restriction enzyme Bam H1.

Inhibition of HSV-1 plaque formation and virus yield reduction assay. Confluent monolayers of Vero cells cultured in 50 mm dia Petri dishes were inoculated with 200 plaque forming units (PFU) of HSV-1 in 0.2 ml DMEM. After 1 hr absorption at 37° the inoculum was decanted and replaced with medium containing 2% (v/v) FCS (maintenance medium), 0.1% pooled human immunoglobulins and increasing concentrations of compounds I and II. After a period of 48–72 hr cells were fixed with methanol and stained with Giemsa. Plaques were enumerated using an inverted microscope. Virus yield measurements were performed on Vero cells infected with 0.1 PFU/cell and exposed to various amounts of the psoralen compounds in maintenance medium. After a period of 48–72 hr from infection, cells were harvested into the culture medium and disrupted by three cycles of freezing and thawing. Virus yields

were finally determined by plaque assay. The amount of substance required to reduce plaque formation by 50% (viral ED_{50}) was derived from the best linear plot of a least square fit relating surviving plaques to psoralen concentration [9].

Cell cytotoxicity. The cytotoxic effect of the psoralen derivatives was determined on exponentially growing Vero and HEp-2 cells by a three day culture experiment. For this purpose cells were originally seeded at a concentration of 5–7 × 10^4 cells in 20 mm dia Costar wells and counted at the end of the 72 hr growth period by a conventional haemocytometer after trypsinization of the monolayers. The overall rate of DNA synthesis was also determined on the same cell lines while in a steady state of growth. Accordingly, confluent monolayers (~2.5 × 10^5 cells/well) were incubated in the presence of increasing concentrations of drugs I and II and 1 μCi/ml ^{3}[H]methyl thymidine for a period of 16 hr. Trichloroacetic acid (TCA) precipitable radioactivity was then measured on cell lysate using a liquid scintillator spectrometer (Packard). Measurements of cell ED_{50} were obtained from the plot relating surviving cells and ^{3}[H]methyl thymidine incorporation to the concentration of psoralens I and II by means of a least square fit.

Incorporation of radiolabelled psoralen into the cells. Confluent monolayers of Vero cells were exposed to a 10 μM solution of tritiated compounds I and II in complete medium . After different periods of time the supernatant fluid was decanted and the cells were extensively washed with an isotonic solution. Monolayers were then lysed, and the radioactivity counted as before. To avoid photoreaction, light exposure was carefully avoided during all culture experiments.

Measurement of viral and cellular DNA synthesis in the presence of compound I. HEp-2 cells were infected with 20 PFU/cell of HSV-1 and incubated with 5 μCi/ml of ^{3}[H]methyl thymidine from 6 to 16 hr following infection in maintenance medium containing a virocidal concentration of drug I, or no drug. After the labelling period cells were lysed with sodium dodecyl sulphate (SDS) and pronase (Sigma). DNA was fractionated by isopicnic centrifugation in a NaI gradient as previously described [10]. Fractions were collected from the bottom of the gradient by means of a peristaltic pump and aliquots were spotted onto GF/C filters (Whatman), to count TCA precipitable radioactivity.

Expression of viral polypeptides in infected cells. HEp-2 cells were infected with 20 PFU/cell and, immediately after the absorption period, exposed to increasing concentrations of compound I in maintenance medium. Cells were then labelled with 10 μCi/ml of a tritiated amino acid mixture in DMEM containing 1/10 of the amino acid concentration and 2% (v/v) dyalised FCS to which the desired amount of compound I was added. The labelling period was extended from 3 to 12 hr following infection in order to cover the bulk of viral polypeptide synthesis. Samples were prepared, and SDS-PAGE carried out as described [11] on a 9.25% slab gel. The polypeptide nomenclature proposed by Honess and Watson [12] was adopted.

Preparation of viral and cellular DNA. Viral and cellular DNA were separated by means of isopicnic centrifugation in a NaI gradient containing 0.5 mg/l of ethidium bromide (Boehringer-Mannheim) to vizualize DNA [10]. Their purification was achieved by conventional methods.

Unless stated otherwise, physical measurements were performed in aqueous 10 mM Tris buffer, pH 7.0, at 25°. The concentrations of DNA, I and II were determined spectrophotometrically using the following extinction coefficients: 6600 M^{-1} cm^{-1} at 260 nm for cellular DNA, 6900 M^{-1} cm^{-1} at 260 nm for viral DNA, 13100 M^{-1} cm^{-1} at 312 nm for I and 11100 M^{-1} cm^{-1} at 302 nm for II.

Spectroscopic measurements. Electronic absorption spectra were obtained on a double beam Perkin Elmer mod. 554 spectrophotometer, thermostatically controlled by a Haake F3C circulating bath. Circular dichroism studies were carried out in a Jasco J 500A spectropolarimeter equipped with a Jasco mod. DP 501 data processor. Four to eight scans were accumulated for each sample.

Equilibrium dialysis. A dialysing membrane (Thomas, U.S.A.) with cutoff 10^4 daltons was sandwiched between two plexiglas half cells (dialysable volume 7 ml). In one part of each cell, the aqueous solution of derivatives I and II was introduced, while the other part contained a DNA solution under identical ionic strength and pH conditions.

Known volumes from each compartment were drawn for a quantitative evaluation of the equilibrium distribution of the drug. In the solution containing the complex, dissociation of the adduct was achieved by addition of equal amounts of 0.2 M lithium chloride in methanol.

Experimental data were plotted according to Scatchard [13] and analysed by the neighbor exclusion model, which describes the binding of a ligand to a homogeneous lattice [14].

$$r/m = K_i (1 - nr) \{(1 - nr)/[1 - (n - 1)r]\}^{n-1}$$

where r is the number of ligand molecules bound per DNA phosphate, m is the free ligand concentration, K_i is the intrinsic binding constant and n the number of consecutive lattice residues occupied by a ligand molecule.

RESULTS

Antiviral activity of I and II. Table 1 reports the effect of compounds I and II on the growth of HSV-1. As far as compound I is concerned, the viral ED_{50} stands between 3.5 and 3.6 μg/ml depending on infectious titre or viral progeny measurements. The very similar inhibition value of viral replication para-

Table 1. Antiviral activity of compounds I and II

Compound	Viral ED_{50} (μg/ml) Plaque reduction	Yield reduction
I	3.5	3.6
II	24.5	n.d.

Table 2. Cytotoxicity of compounds I and II

Compound	Growth inhibition cell lines		Metabolic inhibition cell lines	
	Vero	HEp-2	Vero	HEp-2
I	5.2	6.3	6.8	9.6
II	27.5	25.5	70.1	59.8

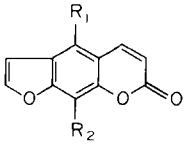
Cellular ED_{50} (μg/ml).

meters is indicating that the majority of particles released during an infectious cycle in the presence of the drug are able to reinfect the host. If this were not the case, and a significant number of defective virions were produced, one should expect the yield value to be somewhat lower than the value of plaque reduction.

Compound II is inhibiting HSV-1 at higher molar concentration (ED_{50} 24.5 μg/ml) and hence has to be considered less active. A 50% inhibition of the viral yield was not attained at doses as high as 20 μg/ml.

Cytotoxicity of compounds I and II. The cells which were used for growing HSV-1, were also employed to determine the cytotoxic activity of derivatives I and II. Inhibition of cell metabolic activity and growth was studied (Table 2). Compound I is more toxic than compound II by an order of 4- to 10-fold according to the biological parameters considered and the cell lines used. A comparative inspection of Tables 1 and 2 shows that the antiviral effect is very poor.

Incorporation of tritiated psoralens into cells. The differences exhibited in the level of antiviral and cytotoxic activity by two structurally very similar compounds might be due to differences in the uptake



I $R_1 = -O(CH_2)_3N(C_2H_5)_2 \cdot HCl$; $R_2 = H$

II $R_1 = H$; $R_2 = -O(CH_2)_3N(C_2H_5)_2 \cdot HCl$

Fig. 1. Chemical structure of compounds I and II.

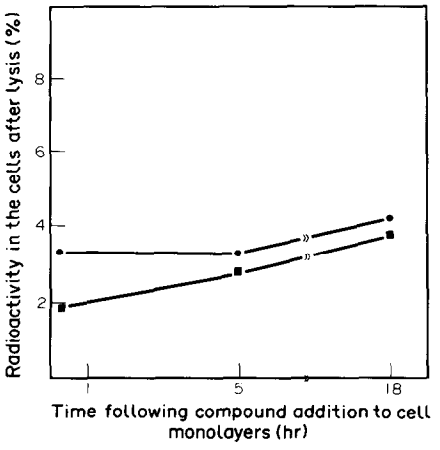


Fig. 2. Incorporation pattern of radiolabelled psoralens I (●) and II (■) into Vero cells.

process. A time course experiment on the incorporation levels into cells has therefore been performed using tritiated compounds I and II at the same molecular concentration. Results are reported in Fig. 2. The drug aliquots persisting within the cell environment after isotonic washing at 5 and 18 hr from addition to cultures are almost the same for both compounds. The different behaviour observed at 20 min is probably due to diffusible drug. Although this approach does not give a true estimate of the cell uptake process, since it does not resolve the amount of diffusible drug, nevertheless it is indicating that incorporation builds up slowly and takes place in a similar way for compounds I and II.

Effect of compound I on isolated viral and cellular DNA synthesis. Only compound I was utilized for this study, as results of previous experiments indicated its higher activity. A drug concentration of 7.5 μg/ml was employed, corresponding to about the cell ED_{50}. Analysis of the density gradient profiles for viral and cellular DNA shows almost complete disappearance of the peak of radioactivity due to incorporation of the radionuclide into viral nucleic acid (Fig. 3, bottom). This result was confirmed in a parallel experiment where ethidium bromide was present in the gradient. No viral DNA was detectable in the treated sample upon exposure to u.v. light. Cellular DNA synthesis in mock infected cells, as expected from the *in vivo* data on incorporation of ^{3}[H]methyl thymidine, is reduced by approximately 50%. It is interesting to note that DNA synthesis in infected cells appears to be completely resistant to the drug action (Fig. 3). The possibility therefore exists that at least two functions connected with DNA metabolism are present (either replicase or repair), which show a variable susceptibility toward the psoralen derivative.

Effect of compound I on the expression of viral polypeptides. Viral protein synthesis was minimally inhibited by drug concentrations up to 5 μg/ml. A remarkable effect was instead noticed at a psoralen concentration of 10 μg/ml, at which viral growth and DNA synthesis were completely suppressed. Inhibition of the expression of viral polypeptides was prominent especially for infected cell protein (ICP) 5, 8, 9, 10, 20, 21 and for β viral protein (VP) 22 and γ VP 23 (Fig. 4). The cellular protein actin, which can be taken as an internal control, is only scarcely inhibited. It is noteworthy that ICP 5 and 21, together with γ VP 23 are polypeptides representative of the late period of synthesis, which has been shown to follow the replication of viral DNA and to depend on it [12].

Thermodynamic binding parameters of I *and* II *to DNA.* A quantitative evaluation of the binding affinity of psoralens I and II to DNA was obtained

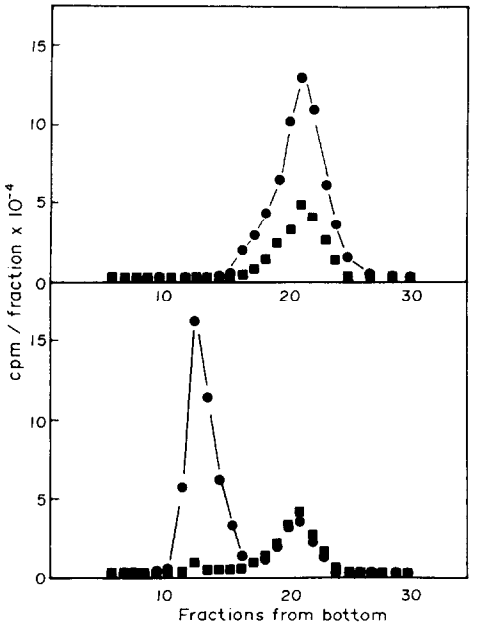


Fig. 3. NaI density-gradient profiles of DNA labelled in uninfected (top) and infected (bottom) HEp-2 cells. (●) no added drug; (■) in the presence of 7.5 μg/ml of compound I.

from equilibrium dialysis measurements. The data are presented as the Scatchard plot in Fig. 5. The results are summarized in Table 3 in terms of intrinsic binding constants, exclusion parameters and binding free energies. It is immediately evident that compound I exhibits considerably higher affinity toward DNA than compound II. The number of available binding sites is also greater for compound II. Supposing a similar dependence of K_i of both drugs on salt concentration [15], we can reasonably assume that the relative affinities of derivatives I and II to DNA are not appreciably modified under physiological conditions.

Conformational studies on the complex of I *and* II *with DNA*. Circular dichroism (cd) measurements were performed for cellular, viral and calf thymus DNA in the presence of compounds I and II at different drug/nucleotide ratios. Addition of increasing amounts of psolaren I causes dramatic changes in the nucleic acid optical activity (Fig. 6). In particular the intensity of the positive band at 275 nm increases sharply as the ligand concentration increases, levelling off when saturation of the binding sites is approached. A similar behaviour is obtained with compound II. These findings along with flow dichroism data (not shown) are consistent with an intercalative mode of binding for both compounds [16–19]. While the examined drugs do not exhibit intrinsic rotational strength, negative cd appears in

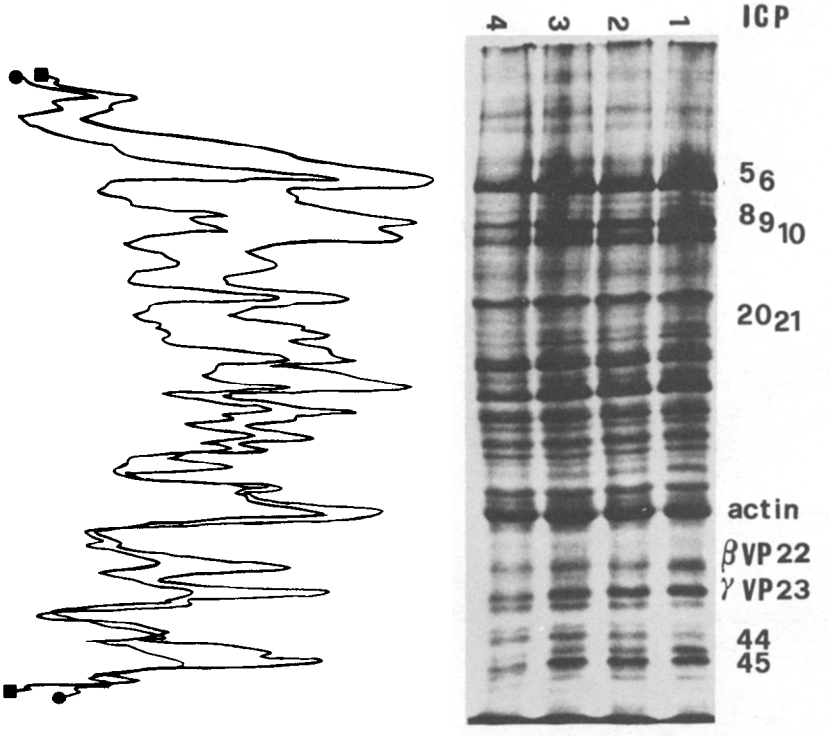


Fig. 4. Autoradiograms and densitometric tracings of labelled polypeptides separated in 9.25% polyacrylamide gel slabs from lysates of HEp-2 taken 12 hr after infection with 20 PFU/cell HSV-1. Lanes from bottom: (1) no added drug; (2) in the presence of 2.5 μg/ml of compound I; (3) in the presence of 5.0 μg/ml of compound I; (4) in the presence of 10 μg/ml of compound I. (■) densitometric tracing of lane 1; (●) densitometric tracing of lane 4.

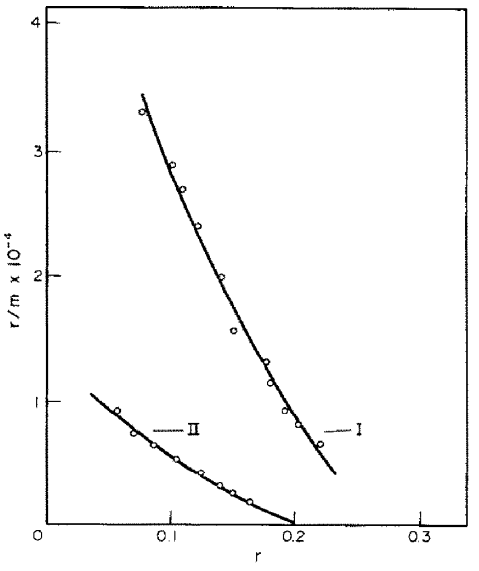


Fig. 5. Scatchard plot for the binding of compounds I and II to calf thymus DNA at 25° in 0.01 M Tris buffer, pH 7.0. Curves I and II refer to the corresponding compounds. Solid lines represent the theoretical plots using the binding parameters reported in Table 3.

Table 3. Thermodynamic parameters for the binding of compounds I and II to DNA in 0.01 M Tris buffer, pH 7.0 at 25°

Compound	$K_i \times 10^{-4}$ (M^{-1})	n	$-\Delta G$ (kJ/mole)
I	5.6	3.1	27.1
II	1.4	3.8	23.7

both cases above 300 nm, when the ligand is bound to DNA. Evaluation of the cd response in terms of DNA structure and orientation of the ligand transition moment [20, 21] suggests that our compounds should intercalate with an orientation similar to acridine orange and ethidium bromide [22, 23].

Interestingly, the intensity of the band above 300 nm differs in the two complexes, being considerably less negative in the case of compound II (Fig. 7).

DISCUSSION

The results reported in the present study show that compounds I and II exhibit an inhibitory effect on the growth of HSV-1 in the absence of irradiation.

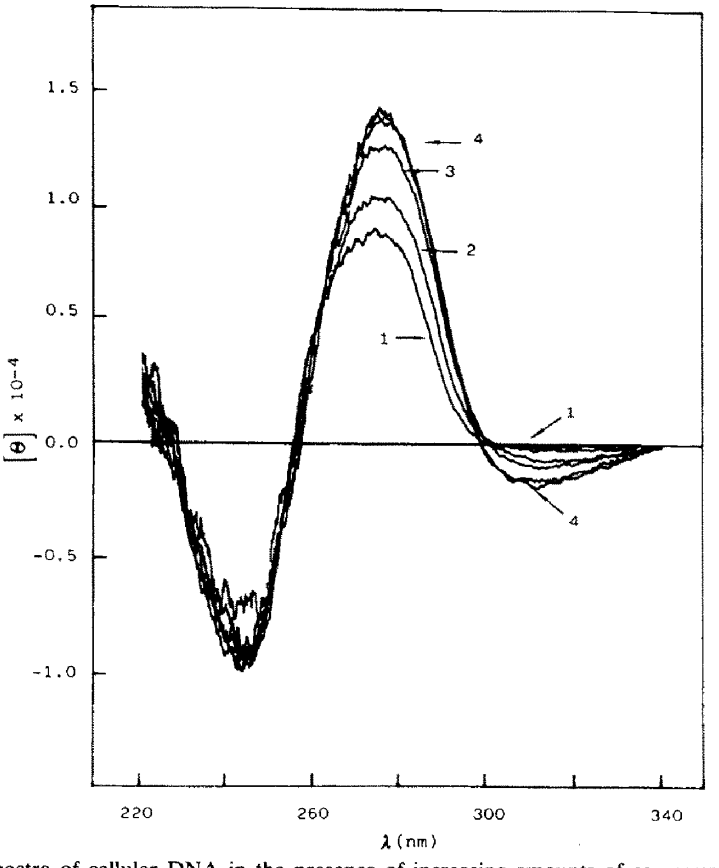


Fig. 6. Cd spectra of cellular DNA in the presence of increasing amounts of compound I at 25° in 0.01 M Tris buffer (R = drug/DNA). $C_{DNA} = 2.0 \times 10^{-4}$ M; (1) $R = 0$; (2) $R = 0.04$; (3) $R = 0.18$; (4) $R = 0.40$.

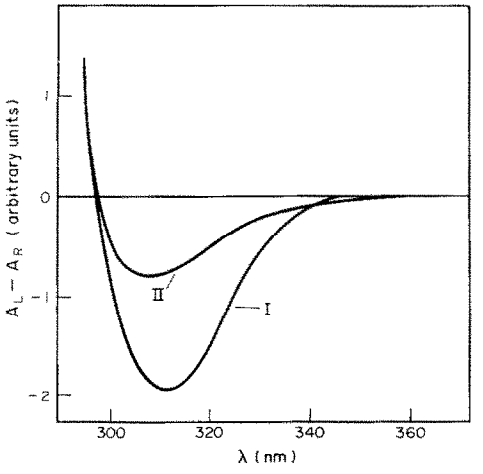


Fig. 7. Cd spectra of the psoralen–DNA complex in the 300–360 nm region. Curves I and II refer to the corresponding compounds.

These findings are at variance with previous literature on other psoralen derivatives [3], for which no antiviral activity was detected.

Compounds I and II are also showing a certain degree of cytotoxicity, which appears to be related to the antiviral effect. The latter can be reasonably considered as a marker of biological activity for the examined drugs.

As far as the mechanism of action is concerned, our results show that DNA synthesis of both cell and virus are remarkably affected upon addition of the drug to cultures. Furthermore a close relationship is observed between inhbition of cell growth and cell DNA synthesis. That DNA is indeed a major target for our compounds is also shown by the preferential inhibition of the expression of late viral polypeptides. The physico-chemical data confirm that the examined drugs exhibit fairly high DNA binding constants, with interaction free energy of the order of −25 kJ/mole.

In spite of their close similarity, derivatives I and II give considerably different biological responses. In particular compound I is more effective both as an antiviral and as a cytotoxic agent. This fact cannot be ascribed to different incorporation into the cell compartment, as shown by drug penetration experiments. On the other hand modified chiroptical responses in the complex absorption region are indicative of somewhat different intercalation geometries into DNA for the two compounds, I behaving closer to the classical model [21]. These findings likely explain the higher affinity of compound I for DNA *in vitro*, and are able to fully account for the results obtained *in vivo*.

Lack of selectivity toward specific polynucleotide sequences is shown by the comparable binding energies of psoralens I and II to DNA from viral or cellular origin. The observed preferential inhibition of viral DNA synthesis should accordingly be related to a modified rate of *de novo* DNA synthesis, rather than to a privileged interaction with the viral nucleic acid. The inability of viral enzymatic machinery to repair damage consequent to strong action on DNA might additionally contribute to the observed phenomena.

The investigation on the effects of a model drug on biological systems and macromolecules from their natural sources can prove quite helpful in elucidating structure–activity relationships. In the present paper it is clearly shown how minor structural modifications dramatically affect the biological response. Physico-chemical studies, on the other hand, give information at the molecular level. Thus a combined analysis of *in vivo* and *in vitro* results, such as the one we report here, represents a powerful tool to design selected modifications of a drug, aimed to specialise and/or potentiate its effects on a particular target. This could not only be the case of psoralen derivatives, but also of the whole family of anti cancer drugs known to act primarily on DNA.

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