Biological, Proton-Magnetic-Resonance- and Ultraviolet-Spectroscopic Evidence for a Molecular Complex of Actinomycin D and 10,11-Dihydro-3*H*-naphth[1,2-*g*]indazol-7-ol

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A proton-magnetic-resonance study of the complex formed between actinomycin D and 10,11-dihydro-3*H*-naphth[1,2-*g*]indazol-7-ol strongly suggests that the indazole lies above the phenoxazine ring of actinomycin D. The complex can be destroyed by addition of dimethyl sulphoxide or dimethylformamide. The actinomycin D-indazole complex inhibits growth of *Pseudomonas fluorescens* and raises the thermal denaturation response of DNA. These data support the hypothesis that a molecular complex is formed which readily inhibits cell growth and interacts with DNA.

Combination chemotherapy has become increasingly important in the chemotherapeutic regimentation of cancer. As a result, understanding the physicochemical properties of molecular complex-formation and the resulting therapeutic activities is of the utmost significance. The role of molecular complexation has found renewed interest in the development of chemotherapeutic models (Korolkovas, 1970). Durham & Keudell (1969) reported that the inhibition of synthesis of amidase (acylamide amidohydrolase, EC 3.5.1.4) by actinomycin D, a clinical anticancer agent, could be prevented or alleviated by 3,4-dihydroxybenzoic acid, and suggested that the two compounds formed a complex that, in this case, inactivated the antibiotic.

A study of selected benzoic acid derivatives that influenced actinomycin D inhibition revealed that the steric specificity and conformational integrity of the molecule were very important. Courtauld models indicated that formation of a molecular complex between actinomycin D and the benzenoid compounds was conceivable, possibly as a result of π -orbital overlap (Durham & Ferguson, 1971). The data suggested that the substituted dihydroxybenzoic acid derivatives had a greater affinity than DNA for actinomycin D. The complex formed between benzenoid compounds and actinomycin D had greater stability than the antibiotic-DNA complex, thus producing a shift in the equilibrium away from the actinomycin D interaction with DNA. A summary of available data relating to the molecular interaction of actinomycin D with model biological systems, most of which are purine and pyrimidine compounds, has been published (Danyluk & Victor, 1970).

A new class of aza steroids (15-aza steroids; 15,16-diaza steroids), used in combination with selected antibiotics, show an enhanced antibacterial activity (Chesnut *et al.*, 1971). Durham *et al.* (1974) reported that 10,11-dihydro-3*H*-naphth[1,2-*g*]indazol-7-ol (compound I) and actinomycin D in combination readily inhibit selected micro-organisms and tissue-culture cell lines.

This paper describes biological and chemical data that support our original hypothesis that the enhanced biological activity of a mixture of the indazole and actinomycin D may arise as a result of formation of a molecular complex.

Experimental

P.m.r. (proton-magnetic-resonance) spectra were recorded at 100 MHz on a Varian XL-100(15) unit equipped with Fourier transform accessories manufactured by Transform Technology Inc. The p.m.r. spectrum of the indazole in [²H]pyridine has been reported (Morgan, 1971). U.v.-absorption spectra were recorded on a Cary model 14 recording spectrophotometer (1 cm light-path).

Pseudomonas fluorescens NND, a Gram-negative

Compound I

chemoheterotroph, was grown in 0.2% succinate minimal medium. Growth experiments were performed in tubes (12mm×150mm) containing 5ml of succinate medium plus test compound(s) and cell inoculum to give a final volume of 6ml. The cultures were incubated at 37°C and cell growth was measured by the change in absorbance at 540nm (Chesnut *et al.*, 1972). Actinomycin D and/or the indazole were dissolved in succinate minimal medium at the desired concentrations just before use.

Studies of the thermal denaturation of DNA were done by supplementing equal portions of *Ps. fluorescens* DNA (isolated by the method of Marmur, 1961) with aqueous solutions of actinomycin D, the indazole, or the actinomycin D-indazole mixture. The influence of the test compounds on the thermal denaturation of DNA was then determined.

Results

Agents such as detergents, EDTA and bacteriophage potentiate the action of actinomycin D by altering the permeability barrier of the cell, thus enhancing entry of the antibiotic into the cell (Leive, 1965; Roy & Mitra, 1970; Reihm & Biedler, 1972). The molecular complex resulting from interaction of actinomycin D with the indazole produces an enhanced inhibition of growth in the Gram-negative micro-organism *Ps. fluorescens* NND (Durham *et al.*, 1974). Actinomycin D (6 μ M) or the indazole (26 μ M) alone produced no change in the growth pattern, whereas the same concentrations, added as a mixture to allow complex-formation, caused complete growth inhibition. A slight delay in the onset of growth inhibition suggests that one site of action may be intracellular, perhaps at the transcription and/or translation level.

Confirmation that the indazole interacts with isolated DNA is noted in thermal denaturation studies and by a decrease in u.v. absorbance of the indazole when mixed with DNA. DNA was isolated and the influence of actinomycin D (16 μ M), the indazole (8 μ M) or the actinomycin D-indazole complex on the thermal denaturation was determined. The actinomycin D-indazole complex raised the thermal denaturation temperature of DNA to 86°C, whereas that of the untreated control was 70°C, the actinomycin D control 74°C and the indazole control 76°C. Thus, not only do actinomycin D and the indazole alone interact with DNA, the two in combination shown an elevated thermal-denaturation value, which supports the hypothesis that the complex interacts with DNA.

Analysis of u.v.-absorption spectra indicated a molecular interaction between actinomycin D and the indazole in aqueous solution (1:1 molar ratio). Actinomycin D (32 μ M) shows a single major absorption peak at 240nm, whereas the actinomycin

D-indazole complex, when measured against a control solution of the indazole, shows an additional absorption peak at 270nm (Durham *et al.*, 1974). Addition of hydrogen-bond-breaking agents (2mol% dimethyl sulphoxide or dimethylformamide in water) to the solution containing the complex caused a loss of the 270nm-absorbing peak. In addition, formation of the complex could be prevented by dimethylformamide or dimethyl sulphoxide if added before mixture of actinomycin D with the indazole.

Published studies at 60MHz on aqueous ($^2\text{H}_2\text{O}$) solutions of actinomycin D strongly supported the earlier postulates (from optical and hydrodynamic studies) that concentrations above 100 μ M resulted in the detection of tetramers that existed in a stacked arrangement (Muller & Emme, 1965; Crothers *et al.*, 1968; Courtois *et al.*, 1968). In contrast, at concentrations from 10 to 100 μ M, side-to-side dimeric structures were considered the more tenable in aqueous solutions.

Our p.m.r. spectra were recorded at 100MHz on a solution containing equimolar quantities (1.32mM) of the indazole and actinomycin D. A deuterium internal lock was used. Because of the exceedingly low solubilities of the compounds in $^2\text{H}_2\text{O}$ the spectra were obtained through 8000 to 15000 accumulations with the aid of Fourier transform analysis. The peaks in the spectra usually do not show the correct intensity ratios because of different relaxation times for different protons (the rate of pulsing was

Fig. 1. Spectra recorded at 100MHz for the indazole (A), actinomycin D (B) and the complex (C)

Spectra were determined with equimolar quantities of the indazole and actinomycin D in $^2\text{H}_2\text{O}$ at room temperature. These spectra were the result of 8000–15000 accumulations with Fourier transform analysis.

Fig. 2. Tentative configuration proposed for the actinomycin D-indazole complex

This model was constructed taking into account such interactions as hydrogen-bonding (-----), a charge-transfer process (.....) and π - π -interactions (|---|), all of which are consistent with the p.m.r.- and u.v. spectral studies.

less than 5T). The aromatic region was poorly resolved. Because of the low solubility of the indazole in $^2\text{H}_2\text{O}$, it was not possible to carry out a study over a wide temperature range. This solubility problem also prevented a concentration study of the indazole and actinomycin D. Therefore only the relevant parts of the aliphatic region are shown from the accumulations.

Fig. 1 (curve A) shows a portion of the spectrum for the indazole. The single peak in the spectrum indicates that the two CH_2 groups have similar chemical shifts in $^2\text{H}_2\text{O}$. The spectrum for actinomycin D is shown in Fig. 1 (curve B); the peaks at -2.9, -2.5 and -2.0 p.p.m. (with respect to sodium 2,2-dimethyl-2-silapentane-5-sulphonate) can be assigned to the 6- CH_3 , 4- CH_3 and threonyl- CH_3 groups respectively (Danyluk & Victor, 1970; Arison & Hoogsteen,

1970). Fig. 1 (curve C) shows the spectrum of the actinomycin D-indazole complex in which the CH_3 peak in the indazole exhibits an up-field shift of 0.2 p.p.m., whereas the positions of other peaks remain essentially unchanged. This must arise from the fact that in the complex the CH_2 groups of the indazole lie above the aromatic part of actinomycin D and experience ring current, whereas the methyl groups of actinomycin D are definitely not in the vicinity of the aromatic part of the indazole.

By assuming a tetrameric species for actinomycin D at the concentrations used in this study, the p.m.r. analysis suggests a similar stacking of the indazole over the π -system of the phenoxazine ring of actinomycin D. However, since the ring methyl protons of the phenoxazine ring are not perturbed, the indazole may be situated to a considerable extent over the peptide portion of the actinomycin D molecule. Nevertheless, the shielding effect on the methylene ring protons in the indazole strongly argues that these protons lie over the π -system of the phenoxazine ring of actinomycin D. Courtauld models indicate this type of complex can be accommodated without immediately obvious electronic or steric difficulties. In view of the disruption of the complex with dimethyl sulphoxide or dimethylformamide (which indicates destruction of hydrogen-bonding), we suggest a reasonable candidate for the actinomycin D-indazole complex in Fig. 2. The pyrazole portion of the indazole may act as an electron donor group (through the electron pairs on the N atoms) in the complex.

Conclusions

The u.v.-spectral data and p.m.r. analysis strongly support formation of a molecular complex between actinomycin D and the indazole. Although the concentrations (approx. 1 mM in $^2\text{H}_2\text{O}$) used in the p.m.r. study were necessary because of analytical limitations, the suggested structure (Fig. 2) may be different from that detected by the u.v.-spectral study (approx. 10 μM). This supposition is based in part on previous work on the structure of actinomycin D in solution (Muller & Emme, 1965; Crothers *et al.*, 1968; Courtois *et al.*, 1968; Arison & Hoogsteen, 1970). Thus, at reactant concentrations of 10 μM , some side-by-side arrangement of actinomycin D and the indazole cannot entirely be eliminated from consideration, but is less defensible when our p.m.r. data are considered. In view of the nearly instantaneous formation of the complex (as evidenced by the immediate appearance of the absorption peak at 270 nm) when actinomycin D and the indazole were mixed, it is not unreasonable to assume that such a complex could be formed and show enhanced biological activity for *Ps. fluorescens* NND in aqueous medium. The enhanced susceptibility of the cells

suggests a more rapid penetration of the permeability barrier by the complex than by either component alone. In addition, the interaction of the complex with cellular DNA indicates that the complex may have considerable stability and may function within the cell as well as at the permeability barrier.

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