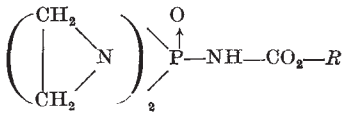
'Dual Antagonists'; Alkyl N-(*bis*-(ethylenimido)phosphoro)-carbamates; a New Series of Anti-Tumour Agents

THE concept of combination chemotherapy in the treatment of neoplastic diseases has been receiving considerable attention during the past few years. Some see the only theoretically possible solution to the problem of cancer chemotherapy in the simultaneous use of several synergistic drugs blocking several consecutive and/or alternative metabolic pathways, while others vigorously object to this thesis, from practical considerations or disappointing experiences.

One of our three collaborating research groups has been particularly interested in the chemotherapeutic potentialities of 'dual antagonists'[1], that is, chemical compounds incorporating the structural features of two different antimetabolites into a single molecule. 'Dual antagonists' are designed to synchronize the action of the synergistic components and thus overcome differences in absorption, distribution, or elimination that would negate or weaken any potentiation arising from simultaneous block of metabolic pathways at several points. If two inhibitors, directed against the same biochemical mechanisms or cellular structures but possessing different degrees of selectivity, are coupled chemically, the more selectively localizing component may prevail in directing the distribution of the molecule as a whole. As a result, the less selective (but often more potent) component may reach concentrations at the desired point of attack that could not be attained by the free (uncoupled) inhibitor below its toxic dosage. In particularly favourable cases, both synergistic components may be released at the desired point of attack gradually and simultaneously, providing for a prolonged local action.

Compound 1 of this communication unites the *bis*-(ethylenimido)-phosphoro radical, a bifunctional group known to have biological 'alkylating' activity, through an amide linkage to urethane, an antimetabolite of nucleic acid biosynthesis[2,3]. The carcinostatic effect of urethane is reported to be synergistic with the similar effect of biological alkylating agents[4].



1, $R = C_2H_5$ ('*AB*-100')
2, $R = CH_3$ ('*AB*-101')
3, $R = CH_2—C_6H_5$ ('*AB*-103')

In compounds 2 and 3, the same alkylating radical is linked to methyl and benzyl carbamate, respectively. Although the latter carbamates, as such, are not known to be effective antimetabolites, it is thought possible that when linked to the electron-attracting phosphoro-group they may split to yield the same active intermediate as urethane.

Compounds 1 and 2 were synthesized by treating dichloro*iso*cyanatophosphine oxide[5] first with ethyl alcohol (or methyl alcohol) and then with ethylenimine in the presence of a hydrogen chloride acceptor. Compound 3 was prepared by heating compound 1 in the presence of benzyl alcohol. All three compounds are white solids which can be crystallized from a mixture of benzene and cyclohexane: m.p.: 1, 88–90°; 2, 119–21°; 3, 134–35° C.

Compounds 1 and 2 are readily soluble in water, but decompose slowly in aqueous solution. In the animal experiments reported below, these two compounds were used in freshly prepared aqueous solutions, while compound 3 was injected in aqueous suspension or, intramuscularly, in peanut oil.

Acute intravenous toxicity tests in *ICR/Ha* Swiss mice gave the following *LD*50 values (mgm./kgm.): 1, 69·4; 2, 62·3; 3, 80·0. The corresponding values after intraperitoneal and subcutaneous administration are the same or only slightly higher, indicating rapid absorption. All deaths occurred between 4 and 9 days after the single injection. Histopathological studies indicated lymphoid involution.

Only a brief outline can be given here of the anti-tumour evaluation of compounds 1, 2 and 3; details will be published elsewhere. Eleven mouse and rat neoplasms were used in this work, and the new compounds were directly compared with nitrogen mustard under a variety of experimental conditions. In doses of equal toxicity, the three compounds were superior or equal to nitrogen mustard. In most cases, compound 3 demonstrated the highest activity, followed by compounds 1 and then 2.

In mice, all three compounds were active in the 'standard' sarcoma 180 test; average *T/C* ratios of 32 per cent by tumour diameter measurement, and 3–4 per cent (0·03–0·04) by tumour weight, were obtained. In different types of experiments with sarcoma 180, *ICR/Ha* Swiss mice were inoculated with known numbers of ascites tumour cells (*a*) subcutaneously and (*b*) intraperitoneally; in all experimental situations, the new compounds caused highly significant inhibitions of (*a*) tumour growth, and (*b*) ascites formation, respectively. Compounds 1 and 3 were also highly effective against Ehrlich ascites tumour and adenocarcinoma 755. In a number of transplanted leukæmias, definite prolongation of life was obtained with compounds 1 and 3; in others, prolongation of mean life-span was of borderline or no statistical significance. Many animals in the latter groups lived much longer than any untreated animals, but many died earlier, presumably because of drug toxicity in a state weakened by disease. This results in increased standard deviation. In *DBA*/2 mice inoculated with leukæmia *L*1210, four animals (out of 50) treated with compound 3 survived 90 days after inoculation and showed no sign of disease when killed and autopsied after this time. In our hands, no other chemotherapeutic agent produced similar apparent cures in any mice inoculated with this leukæmia. In *C3H* mice with 'spontaneous' mammary tumours, highly toxic doses of compound 3 (which actually killed all animals within 2 weeks) caused regression of existing tumours; lower, non-toxic doses inhibited tumour growth.

In rats, all three compounds completely inhibited the growth of Walker carcinosarcoma 256 tumours, at well-tolerated dosage-levels. In Dunning rat leukæmia, all three compounds, administered only during the first 12 days after implantation of the tumours, caused complete inhibition of tumour growth and apparently cured all animals. When they were re-inoculated with the tumour after 42 days, all developed tumours and died with leukæmia. In the control animals, tumours appeared on the eighth day after implantation and all animals died with leukæmia on the eighteenth–nineteenth day. Even when the treatment was delayed until the sixteenth day after tumour implantation, when the average

size of tumour had reached 3·5 cm. and the white-cell count was about 70–90,000, compounds 1 and 3 still caused regression of all visible tumours ; however, in this case, the tumours re-appeared after the treatment was discontinued, and the animals died.

Twenty-five patients with various advanced neoplastic diseases have been treated with compound 1. There was a remarkable absence of gastro-intestinal side-effects. The only side-reaction resembling those of known alkylating agents was depression of bone marrow. It is too early to evaluate the therapeutic results in these patients, although there are indications of anti-tumour effects.

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Fluorescence of Tissue Culture Cells stained with Acridine Orange

Armstrong[1] has shown that biological fluorochrome acridine orange under controlled conditions of *p*H differentiates in fixed tissues betwen deoxyribo- and ribo-nucleic acids, the former showing green fluorescence and the latter red. Schummelfeder, Ebschner and Krogh[2], who examined the nature of this specificity, have shown that acid or heat treatment of fixed tissue, prior to its staining by acridine orange, changes the green-yellow fluorescence of the nuclear matter to a copper-red colour similar to that given by ribonucleic acid in the cytoplasm. From their observations the authors suggested that the green and red shades of fluorescence of acridine orange are associated with high and low molecular-weight forms of the nucleic acids. The deoxyribonucleic acid within the nucleus, however, is characterized by : (*a*) high degree of polymerization and (*b*) high level of orientation, which includes its double-stranded form and the structural organization within the chromosomes. The inquiry conducted by Schummelfeder *et al.* does not indicate the level of depolymerization necessary for the change in fluorescence. It does not also completely eliminate the significance of orientation in the specificity of greenish fluorescence usually shown by chromatin matter within the nucleus stained by acridine orange.

We have made observations on the fluorescence given by some tissue-culture cells stained by acridine orange prior to, and after, their exposure to different dosages of X-rays. Intestine, HeLa, HeLL and conjunctiva cells were grown in basal Eagle's medium on slips in flat test-tubes. After 24 hr. incubation, the slips were transferred to standard veronal buffer (*p*H 7·3) and irradiated with X-rays (International G.E.C., 200 kV., 100 m.amp. ; 160 r./min.) up to a maximum dose of 4,000 r. The cells were fixed immediately in 3 per cent formalin, then transferred to a buffer medium of the desired *p*H, and later stained with acridine orange solution in the same buffer at *p*H 3–6 and concentration 0·05 per cent. The non-irradiated cells were given the same treatment as irradiated ones. The fluorescence of the stained material was observed with blue-violet excitation by using the mercury arc lamp with Reichert blue filter No. 8027 at the source and minus blue filter No. 8006 at the eyepiece.

In the non-irradiated cells all the nuclei showed green fluorescence with nucleoli yellow. The cytoplasm showed crimson red fluorescence. On X-ray irradiation the red fluorescence of the cytoplasm did not show much change except slight intensification. The green-yellow fluorescence of the nuclei also did not show much change until the irradiation dose exceeded 2,000 r. There was, however, a marked change from green towards red when the dose was increased above 2,200 r. and no nucleus with a green fluorescence was left over on the slip, when the dose was greater than 3,200 r. Thus the change of nuclear fluorescence did not appear to be a gradual process but was distinctly confined to a narrow dose-range between 2,000 r. and 3,000 r. These results indicate that in the mechanism that shifts the observed nuclear fluorescence within cells, some step or steps that respond to a cumulative or multihit action of ionizing radiations are involved.

The depolymerization of deoxyribonucleic acid by ionizing radiations has been studied in the solution state by Butler[3], Conway[4] and Daniel, Scholes and Weiss[5]. Their observations indicate that in the aqueous solutions the immediate action on depolymerization of deoxyribonucleic acid by X-rays is very feeble although more significant 'after-effects' are seen. No results concerning the dose effect relationship for the after-effects are yet available. Under the conditions of the present experiments, however, depolymerization studies in the solid state of deoxyribonucleic acid seem to be more important ; for the deoxyribonucleic acid within the cells is considered to be localized within their chromosomal structures, which are often characterized by their gel state containing about 30 per cent solid matter[6].

Observations on the depolymerization of deoxyribonucleic acid in the solid state by ionizing radiations have been reported by Alexander and Stacey[6]. Their studies do not indicate a multi-hit mechanism for the breaks in the main chain of deoxyribonucleic acid. Besides, on the basis of their results, an estimate of the drop in the average molecular weight of deoxyribonucleic acid at 3,000 r. does not amount to even 1 per cent. We are therefore tempted to conclude that the green fluorescence of deoxyribonucleic acid stained by acridine orange is associated with some more labile character, such as its native state, not affected by formalin fixation, rather than simply its high polymer nature.

Beers, Hendley and Steiner[7] have recently shown the formation of two types of complexes between acridine orange and nucleic acids. Complex I involved the bases and inter-nucleotide phosphates of ribonucleic acid. Complex II, however, involves the terminal phosphates of both ribo- and deoxyribo-nucleic acid. They further state that the green fluorescence of nuclei is the result of the formation of complex II with deoxyribonucleic acid and the red