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Perspectives in Cancer Research

Nitrosoureas from Chemist to Physician: Classification and Recent Approaches to Drug Design

JOAN E. McCORMICK and R. STANLEY McELHINNEY
Laboratories of the Medical Research Council of Ireland, Trinity College, Dublin 2, Ireland

Abstract—*Molecular design of chemotherapeutic nitrosoureas is reviewed in the light of a chemical classification of* N-(2-*chloroethyl*)-N-*nitrosoureas* (*CNUs*), *particularly those recently introduced and earlier compounds tested in the clinic. Of the six categories, three are rather arbitrarily based on physicochemical properties: the original, lipid-soluble drugs, water-soluble sugar derivatives, and amides of intermediate character. Others deal with more complex drug designs incorporating antimetabolites* (5-*fluorouracil*), *steroids, redox delivery systems, or hypoxia-selective* 2-*nitroimidazoles. Current attempts to modify the standard* 2-*chloroethyl group, with implications for interstrand cross-linking of DNA, are considered.*

Two unfortunate factors influencing the choice of drugs for clinical trial have been prejudice from the physician and commercial interests. The latter requires no further comment, but a strong plea is made for recognition of the CNU group as one of comparatively few valuable tools for rational drug design requiring appropriate pharmacokinetic evaluation, rather than as a somewhat boring hallmark of repetitive chemists.

INTRODUCTION

THE PAST 30 years have seen the development of several major classes of anti-cancer drugs which are now established as the principal weapons in the armament of the clinical oncologist. The nitrosoureas (NUs) constitute one of these groups, arising from the pioneering work of Montgomery and his colleagues in Alabama in 1963 [1]. Hundreds of congeners of the simple compound *N*-methyl-*N*-nitrosourea, which showed activity against leukaemia L1210, have been prepared and tested, and *N*-(2-chloroethyl)-*N*-nitrosoureas (CNUs) proved particularly efficacious. Of a number of reviews published in monographs and elsewhere, those of Montgomery [2, 3] and Mitchell and Schein [4] are perhaps the most useful on chemical and pharmacological aspects respectively, and of Gibson [5] and d'Incalci *et al.* [6] on the mechanism of action. The present, less comprehensive review attempts a broad, somewhat arbitrary, chemical classification of CNUs, *with emphasis on recently introduced drugs and those which have been used in human disease*, and indicates some trends in present-day NU research at the level of drug design. It formed part of a symposium on CNUs entitled 'The beginning of the end or the end of the beginning?' within the EORTC Pharmacokinetics and Metabolism Group meeting on 'Modern anti-cancer drug development: molecules to man'.* The original outline has now been expanded, documented, and up-dated.

It is appropriate to begin any survey illustrating the progress of anti-cancer drug design during the last 30 years with some reflection on the art and the science of chemotherapy. In this wilderness there are two stark promontories, mentioned in the title of the meeting: man and molecules. On one stands the physician, plying his trade with a considerable degree of artistry. On the other is located what some observers are pleased to perceive as the supreme artist, the organic chemist. The space between is populated perhaps by more artists, the silver-tongued public relations men from the world of commerce, trying for their own reasons to bridge the gulf; and certainly by the cold-blooded scientists—pharmacologists pointing out why the chemist's pride and joy should not be effective in

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the clinic, and screeners triumphantly demonstrating that often they are not very effective anywhere else either. Considerations such as these find frequent examples in the history of CNUs, but the sober judgement is that some drugs have been made recently and will be made in the near future which will prove extremely useful both to the pharmacological scientist concerned with elucidating modes of action and to the clinician concerned with the extent and the quality of the life of his patient. Even limited success of such drugs would of course make speculation on the mental processes of the ancestral organic chemist somewhat irrelevant. So perhaps a judicious blend of art and science has brought us to the end of the beginning.

Urea NH_2CONH_2 is a symmetrical molecule, the diamide of carbonic acid. The nitrogen atoms are designated 1 and 3 or *N* and *N'* in order to distinguish the location of substituents. The drugs effective in cancer chemotherapy carry a nitroso group (−N=O) and a methyl (Me) or a 2(or β)-chloroethyl (CH_2CH_2Cl) group on the same nitrogen atom, i.e. are *N*-methyl-*N*-nitrosoureas (MNUs) or *N*-(2-chloroethyl)-*N*-nitrosoureas (CNUs). Most CNUs which have been developed over the years are *N'*-monosubstituted (Fig. 1, **1**; or **2**, X=H, where a β-hydroxy group is present) and decompose into two very reactive fragments (**3** and **5**, Fig. 1). Isocyanates (**3**, Fig. 1) carbamoylate either intermolecularly (attacking cellular macromolecules such as glutathione reductase) or, in special cases (**3a**, Fig. 1), intramolecularly to form oxazolidinones (Fig. 1, **4**; X=H), although the latter may also be formed by a mechanism involving direct ring closure. Carbamoylation probably makes only a minor contribution to cytotoxicity. The second fragment, 2-chloroethyl diazohydroxide (**5**, Fig. 1), is now established, perhaps rather than a derived carbonium ion (**6**, Fig. 1), as responsible for a major mode of cytotoxic action of the CNUs, alkylative cross-linking of DNA. A guanine residue is rapidly O^6-(β-chloroethylated), and an ethylene (CH_2CH_2) bridge with cytosine on the complementary strand is then formed more slowly by displacement of the β-Cl [7]. In resistant cells (Mer^+) the repair enzyme O^6-alkylguanine alkyltransferase removes ('suicidally') the β-chloroethyl group from guanine before cross-linking can take place [8].

It is convenient to divide CNUs into six categories.

A. *Lipid-soluble*

This property was critical in the development of the prototypical drug BCNU (Carmustine) (**7**, Fig. 2), since it permitted penetration of the blood–brain barrier with apparent implications for tumours of the CNS. Other major drugs CCNU (Lomustine) (**8**, Fig. 2) and its 4-methyl homologue MeCCNU (Semustine) are also in this category [4]. The pyrimidine salt ACNU (**9**, Fig. 2), in regular clinical use in Japan, may also be included in this section since although water-soluble at pH 3 it is much less so under physiological conditions [9].

The cystamine derivative CNCC [10–12] consists almost entirely of equal parts of the isomers (**10** and **11**, Fig. 2). The other symmetrical isomer (**12**, Fig. 2) was actually the target of synthesis but is present in only trace amounts in the obtained nitrosation product, designated CNCC. This lipid-soluble drug has undergone clinical trial in France, but was subsequently replaced by the more active, water-soluble metabolite (**13**, Fig. 2) [12, 13]

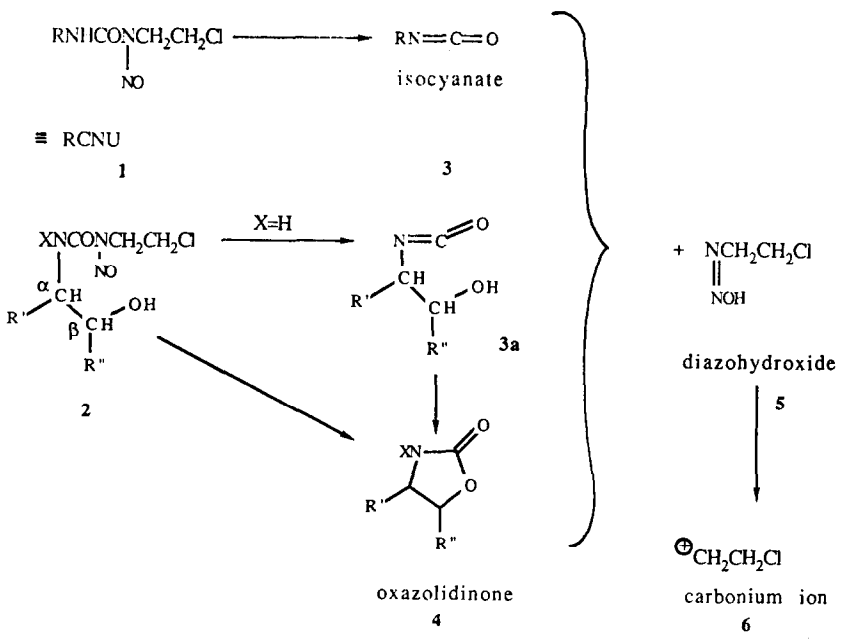

Fig. 1. Decomposition of simple and β-hydroxy-substituted CNUs.

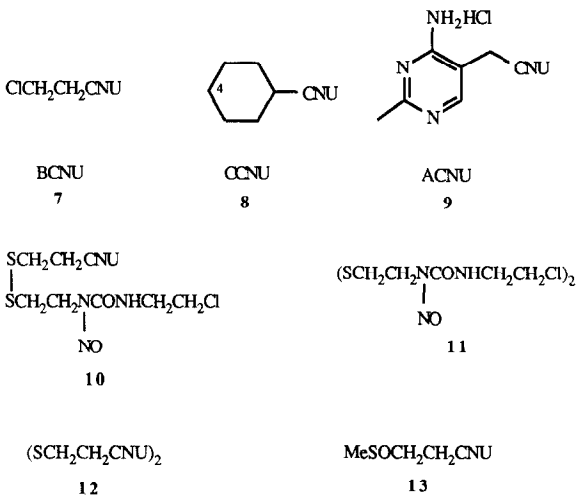

Fig. 2. Some lipid-soluble CNUs and related compounds.

derived together with the corresponding sulphone by rapid *in vivo* disulphide reduction, *S*-methylation, and *S*-oxidation.

B. *Water-soluble*

These are sugar-derived drugs of which the prototype was the naturally occurring MNU streptozocin (**14**, Fig. 3), clinically useful against islet cell carcinoma and malignant carcinoid tumour [4, 14]. Its structure is that of glucopyranose with MNU for OH in the 2-position, i.e. it is a glucosamine derivative. The synthetic analogue chlorozotocin (**15**, Fig. 3) [3, 4, 14a], a CNU of type **2** (Fig. 1), was soon available and showed lower myelotoxicity than the lipid-soluble CNUs. This proved to be due neither to the different physical properties nor to intramolecular carbamoylation of the β-hydroxy group, but possibly to preferential alkylation of DNA in e.g. L1210 leukaemia cells over murine bone marrow and of sites on bone marrow chromatin different from those preferred by CCNU [15, 16]. However, this relative lack of myelosuppression in experimental systems was unfortunately not borne out in the clinic and the drug has now gone out of favour.

Other sugar-derived drugs have been developed principally in Japan and in France. Initially, the effect of locating the CNU group in the unique anomeric position was studied and GANU (**16**, Fig. 3) [17], the 1-CNU isomer of the glucopyranose chlorozotocin (with 2-CNU), and two ribose derivatives were synthesized. The latter drugs were *O*-protected and insoluble in water but readily hydro-

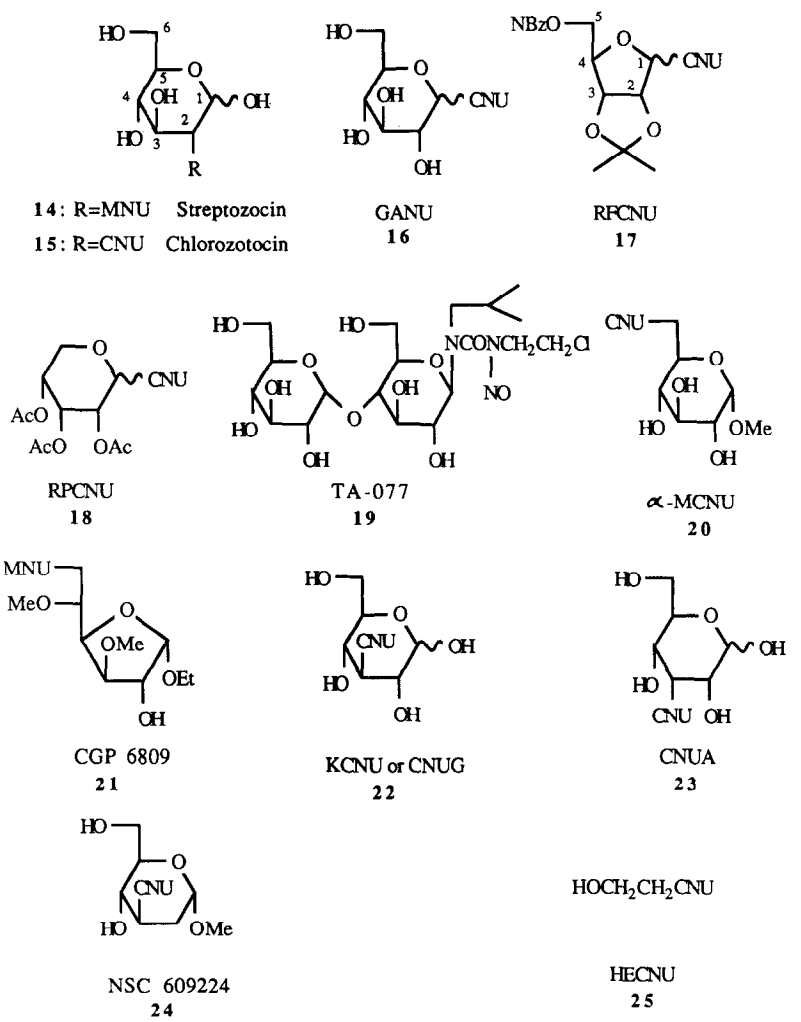

Fig. 3. Sugar-derived or water-soluble CNUs.

lysed *in vivo*: in RFCNU (**17**, Fig. 3) [18, 19] the CNU is attached to 2,3-*O*-isopropylidene-5-*O*-(*p*-nitrobenzoyl)ribofuranose, and in RPCNU (**18**, Fig. 3) [19] to 2,3,4-tri-(*O*-acetyl)ribopyranose. More recently, TA-077 (**19**, Fig. 3) [3, 20], the 1-CNU derivative of the disaccharide maltose with in addition an *N'*-isobutyl group emerged as the most effective of a series of sugar-derived CNUs carrying such *N'*-substituents, i.e. structure **2** (Fig. 1: X ≠ H). In contrast to the common CNUs **1** or **2** (Fig. 1: X = H) which can in theory or in practice generate isocyanates, these *N'*,*N'*-disubstituted CNUs are activated only by direct formation of oxazolidinones (**4**, Fig. 1). Unlike GANU and simpler CNUs, TA-077 showed good activity vs. advanced Lewis lung carcinoma [21], while GANU in turn differed from chlorozotocin in that it was active vs. leukaemia L1210 by the oral in addition to the i.p. route. However, after extensive trials both TA-077 and GANU have been withdrawn as clinical treatments since neither proved significantly better than ACNU. In France, RFCNU was superior to RPCNU in terms of both haematotoxicity and immunosuppression, as well as effective dose range, but interest in CNUs there has largely moved on to the sulphoxide (**13**, Fig. 2) (section A) and those linked to nitroimidazoles (section F).

Following chlorozotocin (**15**, Fig. 3: 2-CNU) and GANU (**16**, Fig. 3: 1-CNU), the terminal carbon (bearing a primary alcohol group in the sugar) of glucopyranose was investigated and MCNU prepared [17, 22]. This drug, the α-methyl glycoside (**20**, Fig. 3: 1α-(*O*-methyl)-6-CNU), differs from other sugar derivatives in not having a β-hydroxy group (as in **2**, Fig. 1) immediately available, although liberation of the 5-hydroxy by hydrolysis would make oxazolidinone formation possible in this case too. It is more active against Lewis lung carcinoma than either chlorozotocin or GANU. MCNU has less hepato- and nephrotoxicity than GANU, but is considerably more myelotoxic than chlorozotocin. It proved clinically useful against haematological malignancies and has gone into commercial production. On the other hand, a more recent glucofuranose with terminal NU group, CGP 6809 (**21**, Fig. 3: 1α-(*O*-ethyl)-3,5-di-(*O*-methyl)-6-MNU) has, perhaps prematurely, been withdrawn before Phase II trial even though a dose schedule at which hepatotoxicity was not serious had been established [23]. Further, unlike the glucopyranose 2-MNU streptozocin this drug is not diabetogenic, and is mutagenic only at very high concentrations.

Of the remaining positions in the glucopyranose ring (bearing secondary alcohol groups in the sugar), no 4-CNUs have been noted, but KCNU (≡ CNUG) (**22**, Fig. 3: 3-CNU) [24, 25] is derived from kanosamine (3-NH_2) in the way that chlorozotocin (**15**, Fig. 3: 2-CNU) is from glucosamine (2-NH_2). In CNUA (**23**, Fig. 3) [26] with the allopyranose structure, the 3-CNU group is inverted with respect to CNUG. While these agents demonstrated good activity against experimental tumours, they have evidently not yet proceeded to clinical trial. In Europe the new glucopyranose drug, NSC 609224 or Acomustine (**24**, Fig. 3: 1α-(*O*-methyl)-2-deoxy-3-CNU, i.e. related to acosamine), apparently neither crosses the blood–brain barrier nor reaches the bone marrow [27]. It has proved very active against several solid tumours including the established Colon 38 adenocarcinoma (known to be resistant to BCNU and many major drugs except 5-FU). Some ribofuranose nucleosides with 3'-NU groups (Fig. 4, **38** and isomer of **39**) [28–30] are mentioned in section C. While all these sugar-derived CNUs are effective anti-tumour agents, it is clear that the location of the CNU group is of considerable significance for the finer details of biological activity. On the basis of these experiments in laboratory animals however it remains difficult to predict the molecular structure that will afford the best therapeutic index in various clinical situations.

The simplest water-soluble CNU is HECNU (**25**, Fig. 3) [31] which is at least as active as other NUs against a range of experimental tumours. Further its long-term toxicity and carcinogenicity are relatively low [3]. It is a simple β-hydroxy-substituted CNU of structure **2** (Fig. 1: X = R' = R'' = H) and oxazolidinone formation prevents carbamoylation of glutathione or of macromolecules such as DNA repair enzymes. A study of HECNU and its isomer $HOCH_2CH_2N(NO)CONHCH_2CH_2Cl$ illustrated the necessity of cross-linking DNA (via chloroethylation) for anti-tumour activity, while single strand breaks (through hydroxyethylation) have merely mutagenic and carcinogenic consequences [32]. HECNU retains lipophilic properties and is very effective against brain tumours in rodents, with considerable promise also shown in the clinic. A strong case can be made for replacement of BCNU as a clinical drug by HECNU. The *N'*,*N'*-disubstituted analogue **2** (Fig. 1: X = CH_2CH_2OH, R' = R'' = H) of HECNU has very high activity against experimental tumours [33], but is an unstable yellow oil.

C. *CNUs with amide groups*

Since amide groups are derived from carboxylic acids and CNUs from amines, the simpler drugs in this section (e.g. **26**, Fig. 4), have amino acids as parent molecules. Their physicochemical properties are intermediate between those in sections A and B. One of the first compounds was PCNU (**30**, Fig. 4) in which CNU replaces the NH_2 group of the cyclic imide of glutamic acid [34]. This drug is very effective in the laboratory but proved disappointing after repeated clinical trials. CNUs from a wide variety of common amino acids [35] and their

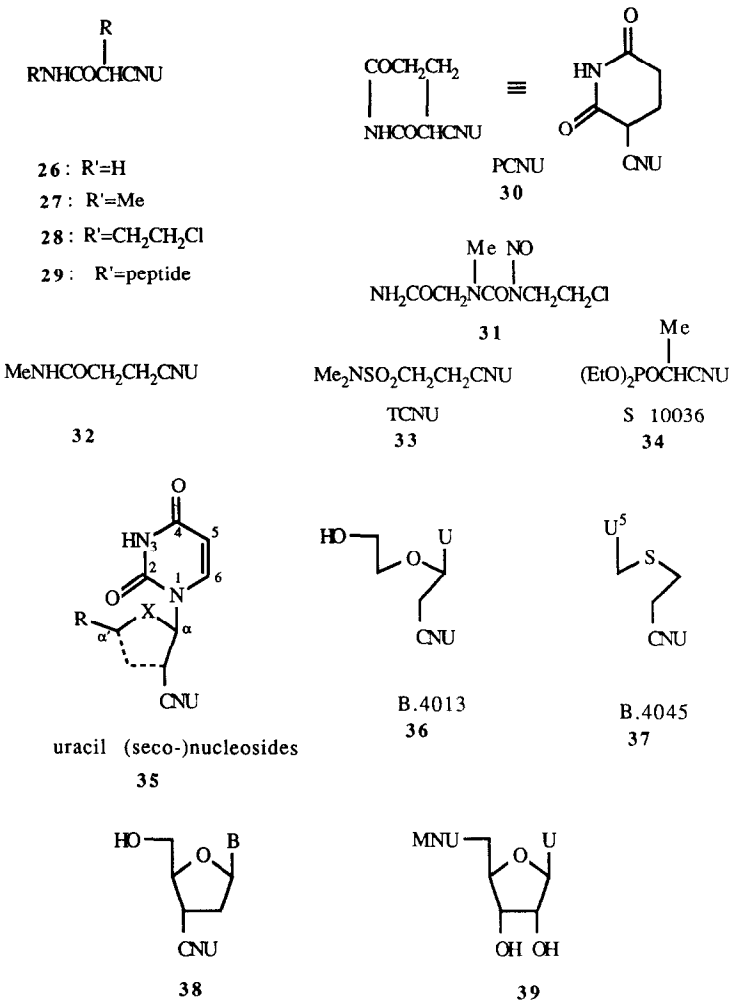

Fig. 4. CNUs with amide and related (seco-)nucleoside substituents.

amides (Fig. 4, **26** [36, 37]; **27** [38, 39]; **28** [40]; **29** [41]) later became available. Sarcosine derivatives (**31**, Fig. 4, and analogue with $ClCH_2CH_2NH$ for NH_2) were among the most effective vs. leukaemia L1210 [37, 40], but the methylamides, e.g. E79 (Fig. 4, **27**; R = Me) from alanine and E149 (**32**, Fig. 4) from β-alanine, were particularly active against solid tumours [39]. The alanylalanine derivative E94 (Fig. 4, **29**; R = Me, 'peptide' = HO_2CCHMe) was toxic to bone marrow only at levels in excess of the maximum tolerated dose and provided some of the very rare cures of the ascitic MAC 15A. As already demonstrated for the sugar residues in section B, the nature of the carrier group is of critical importance in determining toxicity and activity for CNUs, and more pharmacokinetic studies are urgently needed. No clinical trials have as yet been carried out with these amino-acid-derived drugs.

In addition to CNUs from aminocarboxylic acids, useful agents have been derived from aminosulphonic and aminophosphonic acids. The *N,N*-dimethylamide of taurine gives rise to Tauromustine (TCNU) (**33**, Fig. 4), by CNU standards a relatively stable compound which remains in plasma for 8 h following oral administration. It has high activity against refractory solid tumours such as the MAC series [42] and has now reached Phase II trials. Since it closely resembles other CNUs in its reactivity to DNA and related properties [43], its encouraging clinical performance may be a result of pharmacokinetic factors. The drug produced some responses in advanced colorectal cancer [44] and in malignant melanoma [45, 46], and its administration on an out-patient basis, with mostly acceptable side-effects, is advantageous.

Just as TCNU (**33**, Fig. 4) is closely related to the β-alanine drug (**32**, Fig. 4), so alanine drugs like E79 (Fig. 4, **27**; R = Me) have a counterpart in the phosphonic acid derivative Fotemustine (S 10036) (**34**, Fig. 4) [47]. Although it is a lipid-soluble ester rather than an amide, one can classify it in this section. This compound also shows some promise in clinical treatment of malignant melanoma [47] and squamous cell lung carcinoma [47a]. Unlike BCNU, it has the considerable advantage of being a poor inhibitor of glutathione reductase [47b].

Pyrimidines such as uracil containing NHCO groups have this fragment in common with amides, and even though their chemistry is more complex

they tend to have similar physicochemical properties. A number of CNUs containing uracil (U) moieties have been prepared very recently [48] with a view to comparing them with the corresponding 5-fluorouracil (5-FU) drugs (see section D) designed as molecular combinations of antimetabolites and alkylating agents. All these U drugs are seco-nucleosides of general structure (**35**, Fig. 4) in which X = O or S and the pyrimidine may be attached, at the normal (α) or iso-nucleoside (α′) position, by N-1, N-3, C-5 or C-6. Examples are B.4013 (**36**, Fig. 4), with a 'sugar' fragment closely resembling that in the potent antiviral drug Acyclovir, and the seco-ψ-nucleoside B.4045 (**37**, Fig. 4) in which the pyrimidine is attached by C-5 as in ψ-uridine. These drugs show very good activity against solid tumours (MAC colon, and mammary) and the nature of the carrier must be of considerable significance [48]. More orthodox nucleoside derivatives like the 2-deoxyribose analogues (Fig. 4, **38**; B = uracil or thymine) [29, 30] or their isomers in which the CNU and OH groups were interchanged [49] showed the usual activity against L1210 leukaemia, although analogous MNUs, e.g. (**39**, Fig. 4) [28] were largely inactive.

D. *Molecular combinations of CNU and antimetabolites*

The chemical linking, through a carrier component, of a CNU moiety with a second anti-cancer drug to form a chemical or molecular combination illustrates a novel principle for treatment of malignant disease. Two principles of chemotherapy already familiar in the management of clinical cancer can have some advantages over the use of simple or single drugs: (a) gradual release *in vivo* of an active drug from a pro-drug and (b) multiple attack by a (physical) combination of two or more drugs. Examples are Ftorafur (**40**, Fig. 5) and Doxifluridine (**41**, Fig. 5), from which the active antimetabolite 5-FU is split off enzymically; and the treatment of colorectal or cerebral cancer by regimens involving both 5-FU and MeCCNU. Molecular combination offers the possibility of operating these two principles with a single drug. One component of the combination can be released slowly in a manner not possible when two 'free' components are administered.

Nucleosides and seco-nucleosides (**42**, Fig. 5) are now readily available by a new chemical synthesis based on the Pummerer rearrangement [50]. The linear 'sugars' in the acyclic seco-nucleosides (**42a**, Fig. 5) provide a convenient linking device, or carrier, for combining antimetabolite pyrimidines (or purines) (B) with a residue (Y) which can be CNU or other active moiety such as *p*-(3,3-dimethyl-1-triazeno)phenyl (DMTP) [51]. Slow hydrolysis (depicted in Fig. 5) releases the base and a Y-containing fragment (aldehyde), together with the

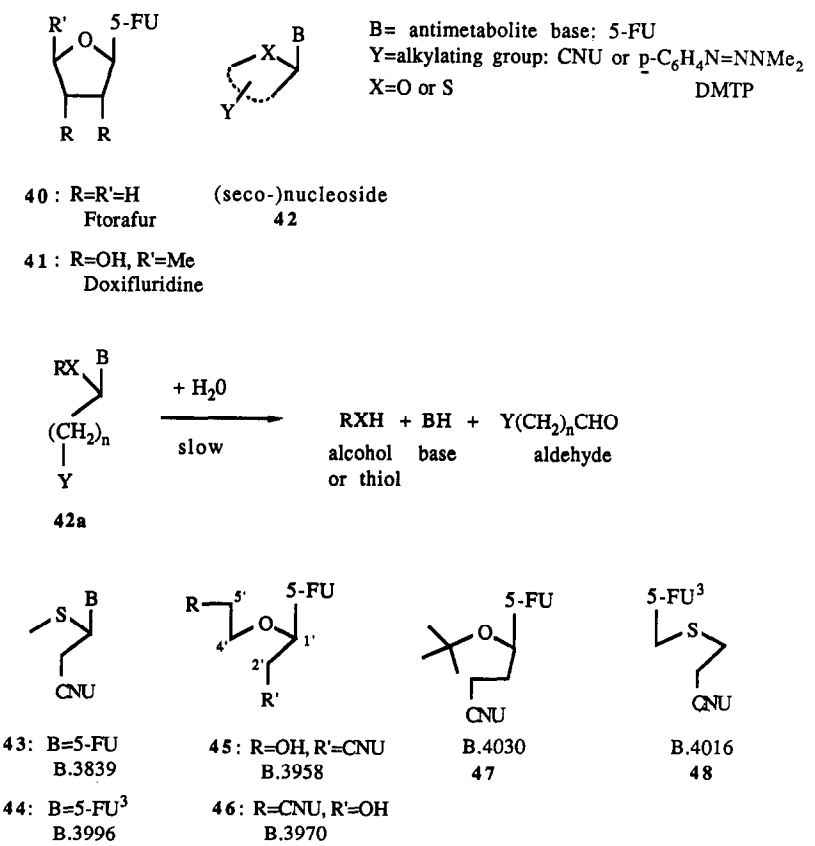

Fig. 5. Pro-drugs of 5-FU and molecular combinations of 5-FU and CNU.

appropriate alcohol or thiol; the residue R may also incorporate a Y group.

This release can be influenced both chemically (stability of bond linking the base, depending on chemical properties of the carrier) and pharmacologically (site of release, depending on absorption and transport, in turn on physicochemical properties of the carrier, e.g. solubility, water–lipid partition). Where a range of molecular combinations of the two active components with different carriers is available, a selected number can be tested in order to pinpoint the type of carrier which will release *in vivo* either enzymically or hydrolytically the latent 5-FU component in such a manner as will, in conjunction with the CNU component, cause maximum damage to a tumour and minimum to the host.

The complex nature of tumours, experimentally induced or spontaneous, growing in animals causes difficulties in assessing the extent to which 5-FU is contributing to the overall effect of these drugs involving both anti-tumour action and host toxicity. Long-term infusion has been advocated [52] as the only rational mode of delivering free 5-FU to patients in order to maintain effective drug levels in plasma, but such administration causes mucositis (which becomes dose-limiting) and patient tolerance is highly variable [53]. Chemical control of 5-FU release could well be preferable to mechanical, and experience with simple pro-drugs of 5-FU such as Ftorafur (**40**, Fig. 5) [54, 55] is very useful for making comparisons with the molecular combinations.

The 'sugar' carrier moiety $RX.CH.(CH_2)_nY$ in (**42a**, Fig. 5) is very versatile and allows the investigation of various chemical and physicochemical factors. Examples of molecular combinations are the prototypical B.3839 (**43**, Fig. 5) [56] and its N^3 isomer B.3996 (**44**, Fig. 5) [57], derived from a thio-sugar; the isomeric alcohols B.3958 (**45**, Fig. 5) and B.3970 (**46**, Fig. 5) in which the 2′ and 5′ substituents are interchanged [58]; the *t*-butoxy derivative B.4030 (**47**, Fig. 5) (McCormick JE, McElhinney RS, manuscript in preparation); and the α′-isomer B.4016 (**48**, Fig. 5) of B.3996 [57]. 2′-Deoxyribose has also been used as a carrier moiety [30].

The molecular combinations show an interesting range of activity against a variety of experimental tumours, and pharmacokinetic studies have begun with a view to correlating these biological properties with some of the (physico)chemical factors discussed above. Thus, the moderate i.p. activity of B.3839 (water-solubility, 1 mg/ml) against the MAC 15A colon tumour (ascites) was lost when the drug was given orally. On the other hand it was inactive against the MAC 13 colon tumour (solid) by the i.p. route whereas oral administration produced good activity. It was highly effective against the Colon 38 adenocarcinoma, for which the isomer B.3996 showed only a slight degree of inhibition. Conversely B.3996 proved one of the most active of these drugs against the MAC tumours, including the refractory MAC26, by both routes of administration, and also strongly inhibited growth of the MCa (Zagreb) mammary carcinoma [57].

The high i.p. activity of the water-soluble (13 mg/ml) B.3958 against MAC 13 disappeared either on oral administration or on interchanging the 2′ and 5′ substituents, i.e. in the drug B.3970 [58]. Both the ether B.4030 (from which 5-FU is released chemically more readily than from e.g. B.3958) and the sulphide B.4016 (slow chemical release of 5-FU) show high activity against the MAC tumours [57]. Prior oxidation of sulphur in the thio-sugar compounds (to sulphones or to relatively toxic sulphoxides) produced much less active drugs [58], although such *S*-oxidation *in vivo* may well be relevant. In general, hydrolytic release (**42a**, Fig. 5) of 5-FU is in the order ether (RO) > sulphide (RS), and N^3-attached B > N^1-attached B, but again the significance of this under physiological conditions has not so far been assessed.

The MAC tumours do not on the whole distinguish between the molecular combinations and their counterparts (section C) carrying U instead of 5-FU, indicating that the release profile of 5-FU, or concentration released at a particular time and site, is probably irrelevant in these systems which are largely resistant to conventional dosage with 5-FU or its pro-drugs. Certain physical combinations of 5-FU and CNUs are advantageous in the treatment of MAC 13 [59], and molecular combination provides a device for exploiting this synergistic effect based on differences in transport and metabolism of single drugs; one example of the significance of drug delivery is the effect of B.3839 on MAC 13 mentioned above and it is hoped that many of those observations will be clarified by pharmacokinetic studies. One notable finding has been that Colon 38 adenocarcinoma, a tumour very sensitive to 5-FU, is largely unaffected by the U analogue (B.3912) of the highly active 5-FU combination B.3839 [48].

Systematic exploration of representatives of the wide range of carriers available may well reveal some release profiles of 5-FU capable of giving a much higher therapeutic index [60] than mere physical combinations. Other experimental tumour systems than those already used may be more appropriate, and in any case the laboratory can at best provide somewhat imperfect models for the ultimate goal of treating slow-growing human tumours. Only clinical trial will eventually tell the real story of the significance of *molecular* combination of 5-FU with CNUs.

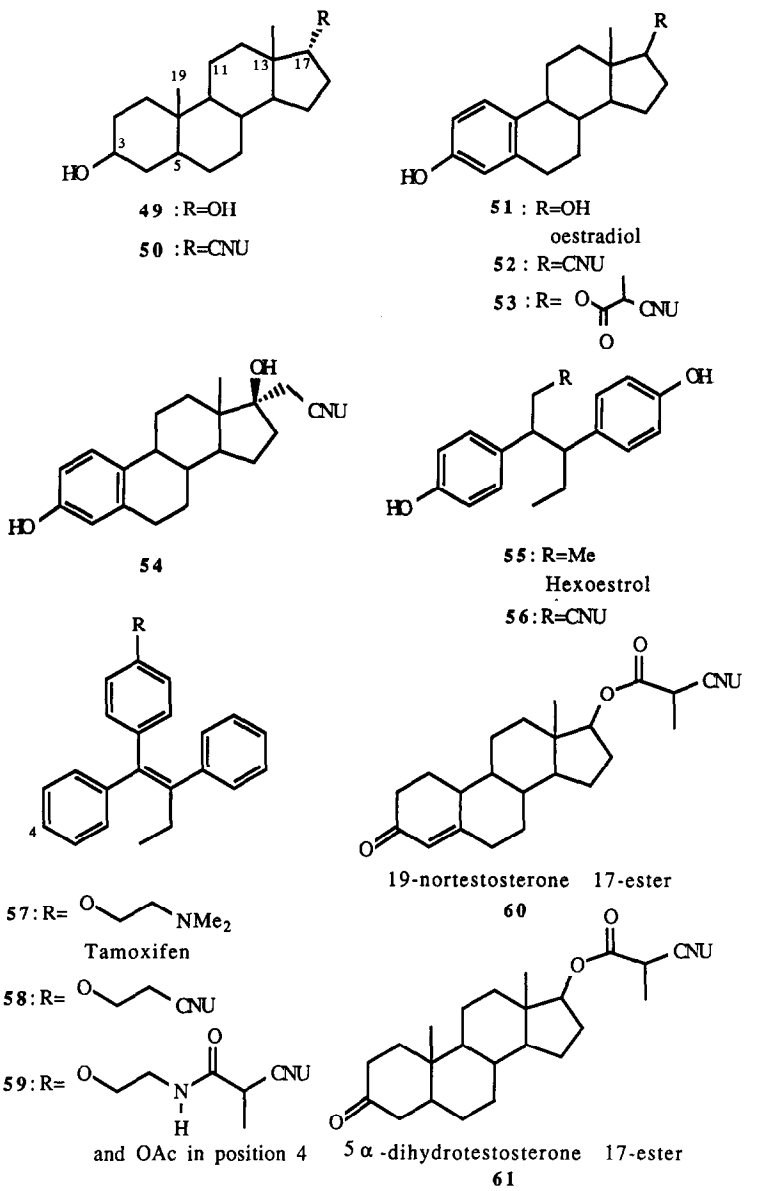

Fig. 6. *Steroidal CNUs and related compounds.*

E. *Steroid-derived drugs*

The first steroidal CNU was the compound (**50**, Fig. 6) analogous to androstane-3β,17α-diol (**49**, Fig. 6). This was made on the basis that the steroid nucleus often imparted high and selective biological activity, and in the event inhibited to some extent the growth of mammary tumour 13762 in rats [61]. Following the discovery that many human tumours are hormone-responsive and contain e.g. oestrogen receptors (ER), the analogue (**52**, Fig. 6) of oestradiol (**51**, Fig. 6) was prepared in the hope that the binding affinity of its steroid moiety for ER might lead to selective accumulation of this CNU in the target tissue [62].

In a similar drug (**54**, Fig. 6) were left free both hydroxyl groups (3 and 17β) of the oestradiol molecule associated with strong binding (through hydrogen bonds) to the ER protein, and in addition a CNU (**56**, Fig. 6) based on the synthetic oestrogen Hexoestrol (**55**, Fig. 6) was studied. Compounds (**52**, **54**, **56**, Fig. 6) showed some specific binding affinity for ER in receptor-positive (MCF-7) human breast cancer cell lines. The order of binding affinity **54** > **52** > **56** > CCNU correlated with the order of cytotoxicity, and the drugs were more active than physical combinations of oestrogen and CCNU. However, their cytotoxicity was non-selective, being very similar against both receptor-positive and receptor-negative (Evsa-T) cell lines, and the order of activity is probably simply a reflection of their stability in aqueous buffer [62].

On the other hand, the CNU analogue (**58**, Fig. 6) of the anti-oestrogen Tamoxifen (**57**, Fig. 6) not only binds to some extent to ER but is more active vs. receptor-positive (MCF-7) than receptor-negative (MDA-MB-231) cell lines [63]. The former

activity is blocked, and the latter unaffected, by oestradiol. However, mediation of the cytotoxic effect via the ER is probably more apparent than real since prolonged treatment is required and the significant factor may be the anti-oestrogenic effect of a hydrolysis product of the CNU (**58**, Fig. 6).

Eisenbrand and co-workers have developed a different type of steroid-linked CNU in efforts to exploit the selectivity possible through hormone receptor binding [64]. Instead of anchoring the CNU group to the oestrogen by a stable linkage, the principle of molecular combination was investigated. This had been developed [56] for CNU groups and the antimetabolite 5-FU, as discussed in section D, where it involved hydrolytic release of a second active cytotoxic moiety. In the present case, a combination of oestrogen and a mobile CNU-containing moiety would be transported selectively to the target tumour for gradual hydrolysis [65]. Amino acids in which CNU replaced NH_2 had yielded the *N*-methylamides (**27**, Fig. 4) considered in section C, and the esterification of such acids with steroidal alcohols now provided an ideal device for constructing these new drugs.

Several amino acids were employed as links between steroid and CNU, most of the biological studies being carried out with the L-alanine esters. Oestradiol (**51**, Fig. 6) for example yielded the 17-ester (**53**, Fig. 6) and the 3-ester (R and OH groups interchanged), both with adequate ER binding affinities. The latter was highly toxic and only moderately active against the MNU-induced mammary carcinoma in rats, but the former (**53**, Fig. 6) has a powerful anti-neoplastic effect (superior to that of the physical combination of oestradiol and the L-alanine-based fragment $HO_2C.CH(Me).CNU$) at relatively non-toxic doses. Moreover, this therapeutic advantage is lost in ovariectomized animals, in which the activity of the ester (**53**, Fig. 6) against the now hormone-independent tumours is only of the same order as the L-alanine-based CNU fragment: this confirms the importance of the presence in the tumours of hormone receptors for successful use of the agent (**53**, Fig. 6) [66].

The work has been extended to androgen receptors, for which the L-alanine esters (**60**, **61** respectively, Fig. 6) of 19-nortestosterone and 5α-dihydrotestosterone proved to have good binding affinity. Compound (**60**, Fig. 6) was particularly effective against the androgen-dependent Noble Nb-R prostate carcinoma in rats, and remarkably less toxic than an equimolar dose of the L-alanine-based CNU fragment [67]. The high activity of compound (**61**, Fig. 6) against receptor-positive (MCF-7) human breast cancer cell lines, measured by the extent of DNA interstrand cross-linking, is strikingly reduced in a competition experiment in which free 5α-dihydrotestosterone is mixed with the drug [68]. Similar effects were observed using mixtures of the ester (**53**, Fig. 6), or of the amide (**59**, Fig. 6) derived from Tamoxifen (**57**, Fig. 6), with the hormone oestradiol. All three drugs (**61**, **53**, **59**, Fig. 6) caused a much lower incidence of DNA cross-linking in receptor-negative (MDA-MB-231) cell lines. Much evidence has thus accumulated that certain steroids can selectively transport the alkylating CNU moiety to tumours containing hormone receptors, and some drugs with a good therapeutic index for these tumours are emerging.

F. *Other recent approaches*

Improved delivery of a CNU-bearing residue to the brain has been achieved [69] by application of a dihydropyridine ⇄ pyridinium salt redox system known to accomplish brain-specific delivery of a variety of drugs ranging from neurotransmitters to antivirals. The active 4-hydroxycyclohexyl metabolite (**64**, Fig. 7) of CCNU could in principle be attached to 1,4-dihydrotrigonelline as the ester (**62**, Fig. 7), although an elaborate synthesis was necessary in practice. The dihydropyridine moiety of this drug was oxidized *in vivo* and the pyridinium form (**63**, Fig. 7) rapidly accumulated in the brain in the manner anticipated. Sustained release of the CNU (**64**, Fig. 7) in this organ followed, while any pyridinium ester (**63**, Fig. 7) was quickly eliminated from systemic circulation. The initial brain levels of compound (**64**, Fig. 7) when it is directly administered systemically are soon depleted. Treatment with the pro-drug (**62**, Fig. 7) could accordingly provide adequate levels of an active CNU in brain tumours while the usual dose-limiting toxicities associated with simple CNUs would be minimized. A similar approach could profitably be explored with the dihydrotrigonelline ester of HECNU (**25**, Fig. 3).

Another recent attempt to improve the performance of CNU drugs resulted in incorporation of the alkylating group in the side-chain of biologically active nitroimidazoles (NI). The hypoxic cell compartment of tumours is the subject of much current attention since it is largely resistant to conventional drug treatment. Compounds such as Misonidazole (**65**, Fig. 7) and analogues with substituents other than OMe at position R have two important effects on these cells: the parent NI rapidly sensitizes them to the effects of radiation, and slower reduction of the nitro group gives rise to an inherently cytotoxic species. These biological properties are selective for hypoxic *vis-à-vis* aerated cells, and a similar selective sensitization to alkylating agents has also been observed.

The drug I-278 (**66**, Fig. 7; type **2**, Fig. 1) was synthesized [70] in order to study the effect of intramolecular chemopotentiation of a CNU. The pharmacology is very complex because in addition

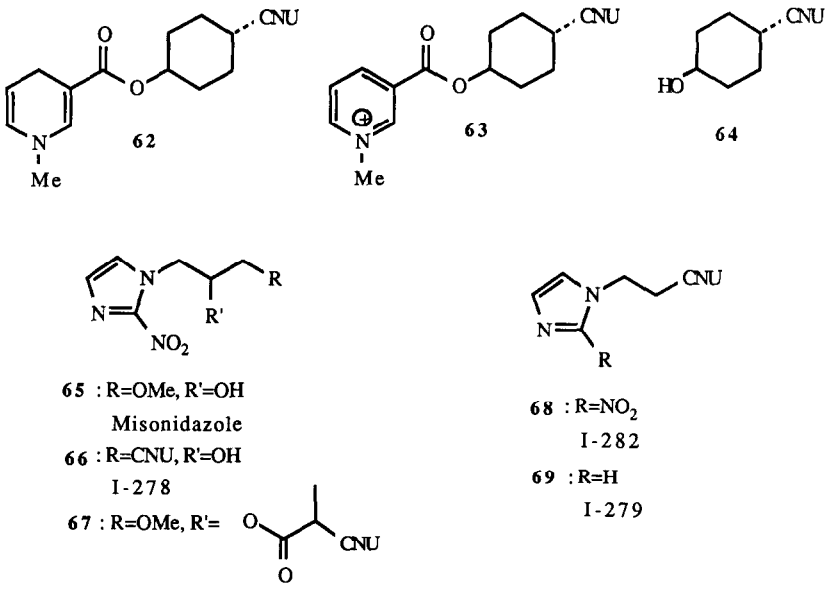

Fig. 7. Some recently designed CNUs.

to the behaviour of the NI fragment in hypoxic cells the alkylating CNU moiety is of course responsible for additional independent cell killing in the aerobic cell compartment, while for compounds like I-282 (**68**, Fig. 7; type **1**, Fig. 1) carbamoylation by the liberated isocyanate represents a further complication. It transpired that the 2-NO_2 in the imidazole nucleus was essential for the exhibition of enhanced hypoxic toxicity against NU-sensitive human tumour cells (HeLa-MR; Mer^-) since only I-278 and I-282 (**66** and **68**, Fig. 7) had such selectivity while I-279 (**69**, Fig. 7) and similar compounds had not. The enhancement was much greater against Mer^- cells than against Mer^+ (HeLa-S3). Further, in the case of Mer^+ the greater effectiveness of I-282 and I-278 is probably associated with the carbamoylating intermediate and its effect on repair enzymes [71].

The enhancement of hypoxic toxicity of the 2-nitro compound I-278 is not due to inherent cell killing by the NI but to chemosensitization to the effect of the CNU [72]. Relatively minute proportions of NI were required to produce the effect intramolecularly, in contrast to the sensitizing of cells to CCNU by potentially toxic doses of Misonidazole. It will be of great interest eventually to correlate these experiments at the cellular level with clinical results.

I-278 (**66**, Fig. 7) is not a molecular combination on a par with structures such as (**42a**, Fig. 5) or (**53**, Fig. 6) which consist of two entities linked by a fissile acetal or ester bond. Rather it resembles such steroidal CNUs as (**50**, **54**, Fig. 6) in which the alkylating group is attached by a stable bond. It would be of interest to test a molecular combination such as the ester (**67**, Fig. 7) which would be the counterpart of compound (**53**, Fig. 6).

Although the 2-chloroethyl group has long been the preferred NU substituent, increasing knowledge of the mode of action has led to some modifications. The relatively slow second stage in ethylene (CH_2CH_2) bridge formation between complementary strands of DNA facilitates competitive dealkylation by the repair enzyme and hence resistance to CNUs in Mer^+ cells. On the basis that more effective interstrand cross-linking might generate greater cytotoxicity, the 2-chloroethyl substituent of CCNU was extended by insertion of a CH_2CH_2S unit to give compound (**70**, Fig. 8) [73]. This device produced an internally activated alkylating species and the drug cross-linked DNA very efficiently, with a 3-thiapentamethylene ($CH_2CH_2SCH_2CH_2$) bridge instead of the ethylene formed by CNUs. However the activity against leukaemia L1210 was still only moderate.

A more recent attempt (McCormick JE and McElhinney RS, manuscript in preparation) to address the question of interstrand cross-linking involves symmetrical bis(NUs) of general structure (**71**, Fig. 8) which in principle generate the bis-alkylating species (**72**, Fig. 8). Examples already recorded were the simple compound (**73**, Fig. 8) which effected some cures of leukaemia L1210 [74], and the series (**74**, Fig. 8) corresponding to CCNU (**8**, Fig. 2) and HECNU (**25**, Fig. 3) respectively, which had moderate or no activity [75, 76]. In the series (**75**, Fig. 8) corresponding to BCNU (**7**, Fig. 2), compounds with $n = 6$ or 7, but not 2, were effective against leukaemia L1210 [76]. The disulphide (**11**, Fig. 2) also belongs to this family, but is relatively inactive [12]. The bis(NUs) (**80**, Fig. 8) isomeric with (**75**, Fig. 8) are of course bis(CNUs) and generate the usual alkylating species together with a bis(isocyanate) (**81**, Fig. 8); they

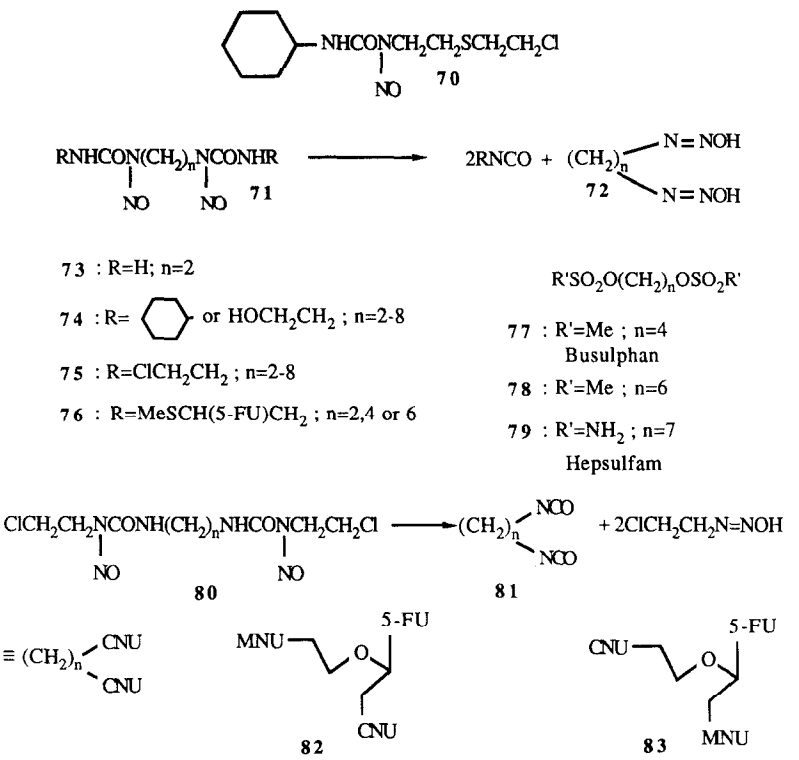

Fig. 8. DNA interstrand cross-linking: polymethylene bis(NUs) and related drugs.

are very active against leukaemia L5222 [31], particularly when $n = 6$.

It seemed appropriate to examine more closely the influence of the two variables, R and n, in (**71**, Fig. 8) on anti-tumour activity. The earlier compounds were tested mainly against rodent leukaemias and it may be that the simple R groups cyclohexyl and $HOCH_2CH_2$ are inadequate when attached to bis(NUs) as in (**74**, Fig. 8). As already noted (sections C and D), the complex seco-nucleoside groups incorporating either U or 5-FU significantly influence drug transport and are very good carriers of CNU, often yielding agents that perform better than the simple CNUs, so compounds (**76**, Fig. 8) have been synthesized which allow direct comparison with B.3839 (**43**, Fig. 5, i.e. MeSCH(5-FU)CH_2CNU).

By varying n in (**76**, Fig. 8), i.e. the length of the anticipated polymethylene bridge, it is hoped to determine whether there is an optimal geometric factor for cross-linking DNA. It is of interest that although the polymethylene bis(methanesulphonate) Busulphan (**77**, Fig. 8) has long been established in treatment of chronic myeloid leukaemia, the higher homologue (**78**, Fig. 8) was later shown to cross-link DNA more effectively [77]. This is also true of the bis(sulphamate) Hepsulfam (**79**, Fig. 8) which has very recently been entered for Phase I trial in the U.S.A. [78].

In a different, unsymmetrical type of bis(NU), regiospecific location of nitroso groups has been accomplished to provide a synthesis of the complex molecular combination (**82**, Fig. 8) and its isomer (**83**, Fig. 8) [79]. These are being compared with the corresponding pair of CNU/OH isomers (**45**, **46**, Fig. 5) (section D) and their capacity for cross-linking DNA is being investigated.

CONCLUSION

At the outset the view was expressed that CNU research has reached the end of the beginning. One factor in this judgement is the status of the CNU group. If variations on the CNU theme are of the ethyl-for-methyl-in-the-carrier-moiety type, perhaps the clinician has some justification for an upturned nose at 'just another CNU'. But while the number of such variations is in principle unlimited, the number of key groups like CNU is certainly not unlimited and probably quite small.

Only a handful of (partly) successful synthetic anti-cancer drugs have been designed over the past 30 years. Many plausible molecular devices have been investigated biologically and found wanting—illustrated most recently by attempts to construct hypoxia-selective chemosensitizing drugs [80] based on groups other than quinonoid or heterocyclic nitro or *N*-oxide. Accordingly the CNU group should be regarded as the powerful tool it is, especially now that much is known of its mechanism of action, and when it is deliberately incorporated into molecules such as are described in this review careful and unbiassed consideration based on detailed pharmacokinetic study should be given to possible clinical trial.

The alternative to discovering powerful new drugs by molecular design and synthesis is by rummaging in Nature. Recent successes like taxol [81, 82] from the forest and bryostatin [83] or dolastatin [84] from the ocean attest the increasingly exotic sources and the scarcity of material; in one case a whole species (a slow-growing yew tree) is threatened, in the others minute quantities of active compounds have been harvested from sea-mats and sea-hares. The chemistry has of course proved fascinating, both structure determination and attempts at (partial) synthesis, but it is so complex that its relationship to the observed anti-cancer activity will not easily be unravelled, and further progress with naturally occurring drugs will probably depend on the emergence of the next esoteric molecule.

On the other hand the interaction of groups like CNU with nucleic acids and other cellular components should become increasingly clear, facilitating the discovery of synthetic drugs with improved therapeutic index [60]. Pharmacokinetic and clinical study of some of the compounds in this review should lead to further significant advances in the chemotherapy of cancer.

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