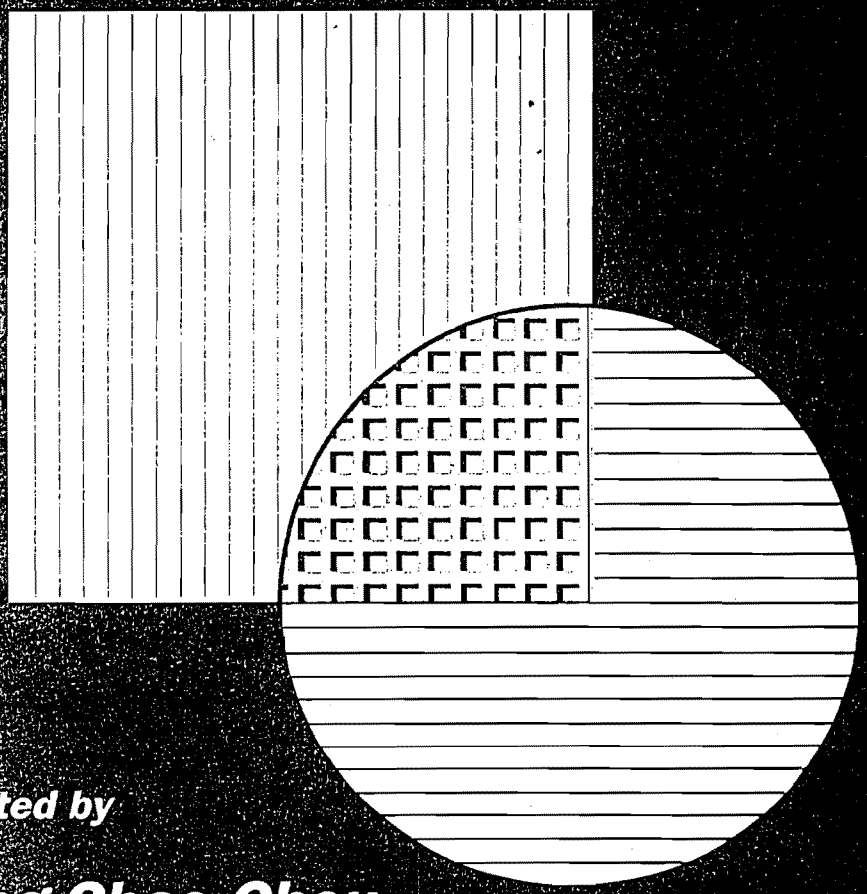
Synergism and Antagonism in Chemotherapy



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CHAPTER 14

Synergism and Antagonism through Direct Bond Formation between Two Agents in Situ

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SYNERGISM AND ANTAGONISM IN CHEMOTHERAPY

I. Introduction

The simplest possible mechanism for synergistic or antagonistic interaction between two agents involves chemical bond formation between the agents *in vivo* to produce an adduct with altered biological properties. This mechanism can be described by the term "covalent modulation" in the sense that agent A modulates the activity of the agent B by forming a covalent bond with agent B. If the adduct has a greater cytotoxic or antimicrobial effect than the precursors (prodrugs) from which it is formed, the combination is known as a "self-assembling drug" (Rideout *et al.*, 1990a). Because of its relative simplicity, covalent modulation has inherent advantages over other mechanisms for the rational design of combinations exhibiting target-selective synergism or host-tissue-selective antagonism. Both of these properties can result in a combination of agents with an enhanced therapeutic index relative to the individual agents or the preformed adduct.

This chapter discusses nonbiochemical, bimolecular reactions that can occur under nearly physiological conditions and *in vivo*, mechanisms for the enhancement or inhibition of bioactivity in bifunctional molecules relative to the corresponding monofunctional molecules, and synergism and antagonism involving direct covalent combinations as a mechanism of interaction. Leading references and illustrative examples will be provided without attempting to comprehensively review the literature.

This chapter does not discuss nutritional synergism involving covalent bond formation *in situ* between drugs and naturally occurring nutrients or catabolites. For a discussion of antimalarial synergism involving combination of naturally occurring metal ions and chelating drugs, see Chapter 5 by Vennerstrom *et al.*

II. Nonbiochemical Condensation Reactions Occurring *in Vivo* or under Near-Physiological Conditions

A. HYDRAZONE FORMATION

Hydrazone formation from aldehydes (or ketones) and hydrazine derivatives (Fig. 1) is generally rapid in water and, in contrast to imine formation, is often irreversible (see Rideout *et al.*, 1990a and references therein). Hydrazone formation has been observed in animal and human subjects. For example, hydralazine reacts with biogenic aldehydes and ketones such as acetone, pyruvic acid, and pyridoxal in human subjects (O'Donnell *et al.*, 1979).

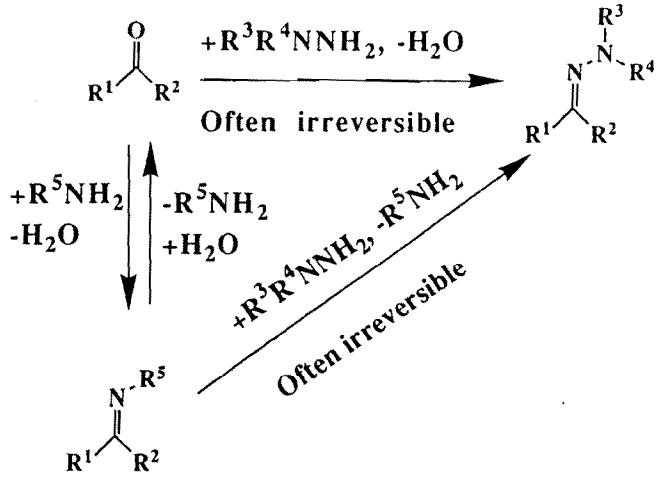


FIGURE 1 Hydrazone formation in water.

Aminoguanidine reacts in animals with the Amidori product, a ketone, which is formed from glucose and proteins. Because of this hydrazone formation, aminoguanidine is useful in preventing protein cross-linking that is responsible for many of the medical problems plaguing diabetics (Ledl and Schleicher, 1990). Aminoguanidine is currently undergoing clinical trials for the treatment of diabetes. Hydrazino-aminovaleric acid forms a hydrazone with pyridoxal bound to ornithine decarboxylase in live mammalian cells (Hölttä *et al.*, 1981).

Aldehydes, hydrazine derivatives, hydrazones, and ketones are all biocompatible. The clinical anticancer agent cyclophosphamide is converted to its active form, an aldehyde, in human liver (Kwon and Borch, 1989). Leupeptin, a peptide aldehyde which inhibits proteases only in its aldehyde form, can be administered to mice and exhibit protease inhibition *in vivo* (Salminen, 1984). Benzaldehyde, which occurs naturally in figs and almonds, has been reported to possess anticancer activity against P388 leukemia in mice and (in prodrug form) against a variety of tumors of human subjects (Balazova and Koza, 1988; Kochi *et al.*, 1980). One of the active metabolites of the antineoplastic agent hexamethylene bisacetamide formed in human subjects is an aldehyde, 6-acetamidohexanal (Subramanyam *et al.*, 1989). Clinically useful monofunctional hydrazines include hydralazine, isoniazid, phenelzine, and carbidopa (Gilman *et al.*, 1980). Hydrazine sulfate itself has been tested clinically against cancer cachexia (Chlebowski and Heber, 1986; Chlebowski *et al.*, 1987). It is interesting to note that hydrazide derivatives of oligodeoxynucleotides have been prepared. These have been useful in the preparation of stable enzyme/nucleic acid hydrazones as hybridization probes (Ghosh *et al.*, 1989). Peptide hydrazides and peptide aldehydes have also been described (DeGrado and Kaiser, 1980; Salminen, 1984). For examples of biocompatible hydrazones and ketones, see Huff (1989) and Smith *et al.* (1983).

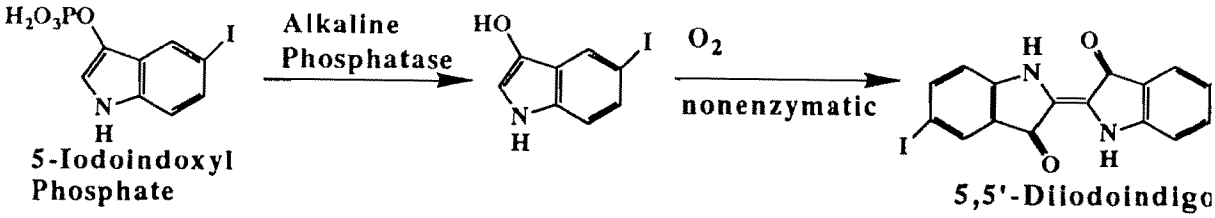


FIGURE 2 Diiodoindigo assembly from an iodoindoxyl ester.

B. INDIGO FORMATION THROUGH OXIDATIVE INDOXYL DIMERIZATION

It is possible to form 5,5′-diiodoindigo inside HeLa human cervical carcinoma cells by administering a monomeric precursor, 5-iodoindoxyl phosphate. The reaction occurs through the mechanism shown in Fig. 2, involving dimerization through nonenzymatic carbon–carbon double bond formation (Tsou, 1968). Cells thus treated become sensitized to treatment with laser light. Similarly, selective formation of 5,5′-diiodoindigo in esterase-rich ciliary body rabbit eyes is achieved by administering 5-iodoindoxyl acetate. It was suggested that 5-iodoindoxyl acetate might by useful for laser eye surgery, since selective destruction of the ciliary body by laser light is possible in eyes treated with this agent (Vucicevic *et al.*, 1969). Indigo is believed to form from indoxyl sulfate in the human bladder through analogous chemistry: sulfate ester cleavage and oxidative dimerization (Dealler *et al.*, 1988).

C. DIELS–ALDER REACTION

Diels–Alder reactions (example in Fig. 3) between lipophilic dienes and lipophilic dienophiles can be substantially more rapid in water and in aqueous surfactant suspensions than in organic solvents because of hydrophobic interactions between the two reactants (Rideout and Breslow, 1980; Breslow *et al.*, 1983). It has been suggested that nonenzymatic Diels–Alder reactions are involved in the synthesis of certain polycyclic natural products (Carruthers, 1978; Joshi *et al.*, 1975). Thus, Diels–Alder reactions that occur *in vivo* might be useful in terms of achieving synergism or antagonism through direct combination of dienes and dienophiles.

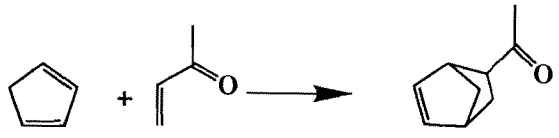


FIGURE 3 A Diels–Alder reaction.

D. METAL–LIGAND BOND FORMATION

The complexation reaction between nickel dication and pyridine-2-aza-*p*-dimethylaniline (Fig. 4) is of interest because it is catalyzed by a number of anionic surfactants. The extent of catalysis varies dramatically with the length and number of lipophilic chains in anionic surfactant micelles, suggesting that reactions of this sort might be catalyzed by biological membranes which contain anionic head groups (Jobe and Reinsborough, 1984; see Section V for example of antagonism involving metal complexation). The intercalators ethidium and proflavine can be linked to DNA using cisplatin: platinum–nitrogen bonding is involved (Malinge and Leng, 1986). When ethidium, cisplatin, and DNA are combined, an ethidium/cisplatin/guanine covalent complex forms (Malinge *et al.*, 1987). It was suggested that this type of self-assembly between cisplatin and amino intercalators might be responsible for a certain type of synergism involving cisplatin in combination with other DNA-binding antineoplastic agents. A structure of an ethidium/cisplatin complex responsible for the lesion has been attained using X-ray crystallography (Sundquist *et al.*, 1988). The reaction is quite specific: although DNA promotes combination between cisplatin and ethidium DNA does not promote combination between transplatin and ethidium nor between acridine and ethidium (Malinge and Leng, 1986; Sundquist *et al.*, 1988). The interactions between cisplatin and aminointercalators may well involve complexation of one or both drugs to the DNA *before* assembly (see Chapter 12 by Strekowski and Wilson).

E. ALKENE PHOTODIMERIZATION

As in the case of Diels–Alder reactions, photodimerization of alkenes can be significantly more rapid in water and aqueous surfactant solutions than in organic solvents because of hydrophobic interaction between the reactants.

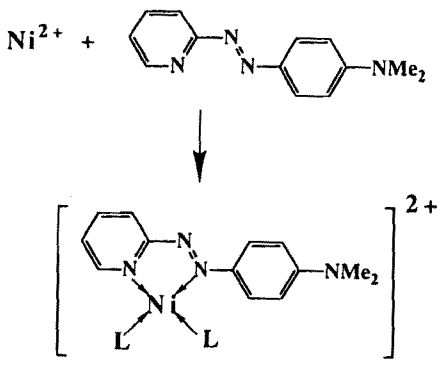


FIGURE 4 Metal chelate formation between nickel dication and pyridine-2-aza-paradimethylaniline.

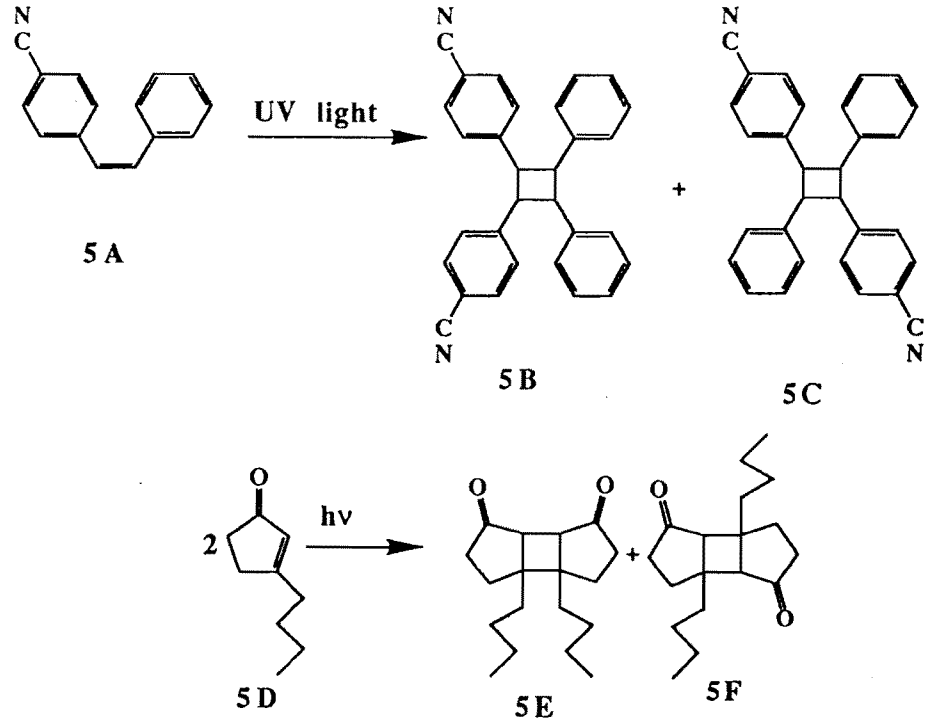


FIGURE 5 Photochemical alkene dimerization to form cyclobutanes.

Photochemical dimerization of the stilbene derivative **5A** at 0.2 m*M* concentrations, as shown in Fig. 5, is significantly more efficient in water than in organic solvents. The dimerization yield (for **5B** + **5C** combined) is 80% in water, yet only 4% in methanol. In contrast, only traces of the dimerization product are formed in benzene. These differences have been attributed to the hydrophobic interactions between pairs of stilbene molecules in water (Ito *et al.*, 1989). Photodimerization of 5.9 m*M* **5D** in benzene yields only about 0.01% **5E** and 0.9% **5F**, whereas dimerization of 5.6 m*M* **5D** in aqueous anionic surfactant micelles yields 98% **5E** and 2% **5F** (Lee and deMayo, 1979). Thymine dimer formation at TT sequences in DNA, a form of alkene photodimerization, can occur in living cells (Kornberg, 1980). Together these results suggest that photochemical cyclobutane formation from pairs of alkene prodrugs should be feasible *in vivo*.

III. Enhanced Cytotoxic and Antimicrobial Bioactivity of Polyfunctional versus Monofunctional Molecules

It is not at all difficult to decrease the bioactivity of a molecule by chemically linking it to a second molecule. If a bifunctional molecule C is formed by linking a monofunctional molecule A to a part of a monofunctional molecule

B that is critical for the binding of B to its receptor, the relative bioactivity of the bifunctional molecule C will be diminished or eliminated altogether. If A and B can combine under physiological conditions to form C, and B is more bioactive than A and C, the result will be antagonism between A and B. (For examples, see Section V.)

In order to achieve synergism through direct combination, however, the bifunctional or polyfunctional adduct must be more active than either of the two monofunctional adducts from which it forms. Although the development of bifunctional molecules that exhibit enhanced bioactivity relative to their monofunctional counterparts is not always straightforward, there are still very many examples of such bifunctional molecules. Several representative examples of such bifunctional molecules will be discussed in this section.

A. DNA BISINTERCALATORS

Molecules formed by linking two monointercalators to one another by a long tether are frequently much more avid DNA binders than the monointercalators themselves because the dimers are capable of bisintercalation (Wirth *et al.*, 1988; Jaycox *et al.*, 1987; Kuhlmann and Mosher, 1981; Delbarre *et al.*, 1987). Often, the dimeric intercalators are much more cytotoxic against cultured tumor cells and/or have greater anticancer activity in animal models compared with the corresponding monointercalators. For example, aminoacridine **6A** shown in Fig. 6 inhibits L1210 leukemia with an ID_{50} of 2.3 μM whereas the ID_{50} values are 0.4 and 0.081 for bisintercalators

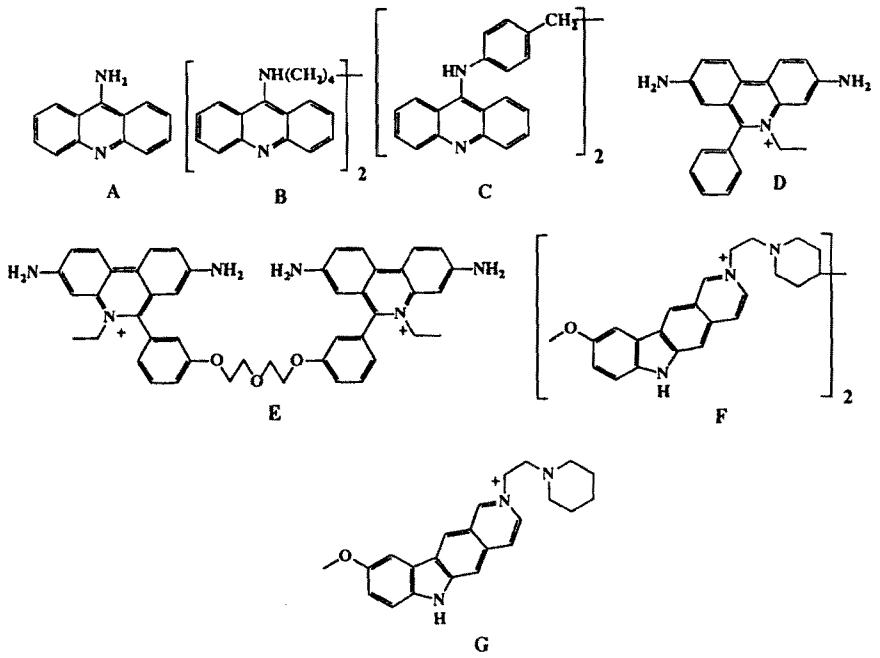


FIGURE 6 Monointercalators and bisintercalators.

6B and **6C**, respectively (Jaycox *et al.*, 1987). A dose of 8.9 μmol/kg of ethidium chloride **6D** is required to extend the life span of L1210 bearing mice by 50% whereas only 1.7 μmol/kg of the related bisintercalator **6E** is sufficient to extend the life span of these mice by 50% (Kuhlmann and Mosher, 1981). The bisintercalator ditercalinium (**6F**) dissociates from DNA 100–1000 times as slowly as the monomer **6G** (Delbarre *et al.*, 1987). In the case of **6F** and **6G** there is also a striking difference in mechanism of action. Ditercalinium (**6F**) causes the complete disappearance of mitochondrial DNA in L1210 leukemia cells without any observable effect on nuclear DNA. In contrast, **6G** only affects the nuclear DNA, does not have an effect on mitochondrial DNA content, and is less cytotoxic than 6F (Segal-Bendirdjian *et al.*, 1988; Fellous *et al.*, 1988). DNA binding constants can increase enormously as the number of intercalating units in the molecule is increased in certain cases. Estimated DNA binding constants for monoacridine (**7A**), bisacridine (**7B**), and trisacridine (**7C**) (Fig. 7) to DNA are 3×10^6, 2×10^{11}, and 10^{14}, respectively (Laugaa *et al.*, 1985). Daunorubicin (**8A**) in Fig. 8 can extend the life span of mice infected with P388 lymphocytic leukemia by only 71% at its optimum dose, whereas the dimeric hydrazones of daunorubicin (**8B** and **8C**) can each extend the life span in the same system by more than 108% at their optimum doses (Henry, 1982).

B. BISALKYLATING AGENTS

Bisalkylating agents often exhibit significantly better antineoplastic activity than monoalkylating agents, by virtue of the ability of bisalkylating agents to form cross-links between DNA strands. A number of bisalkylating agents, including cyclophosphamide and mechlorethamine, are used clinically in cancer treatment (John, 1983; Gilman *et al.*, 1980).

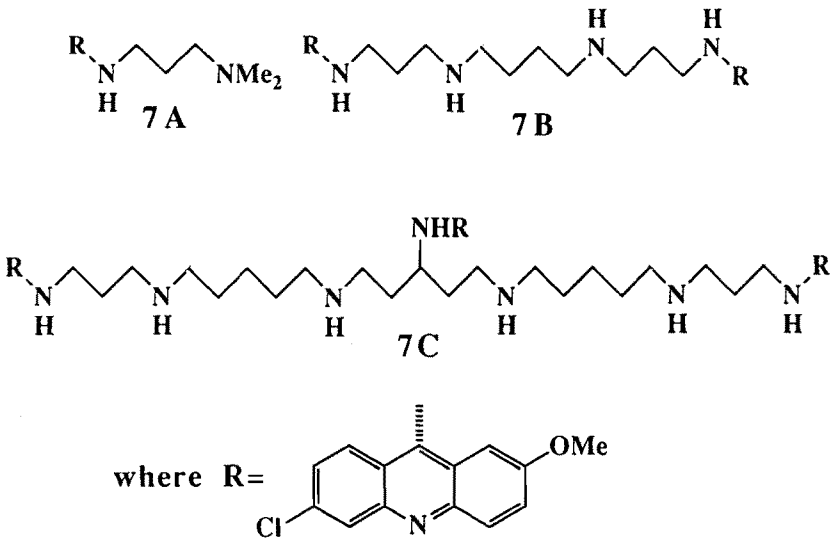
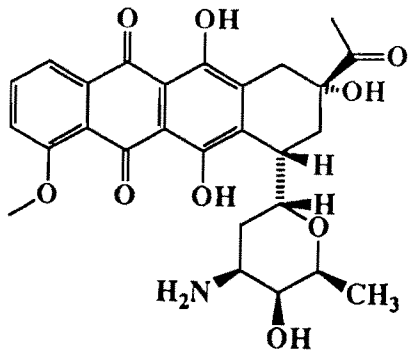
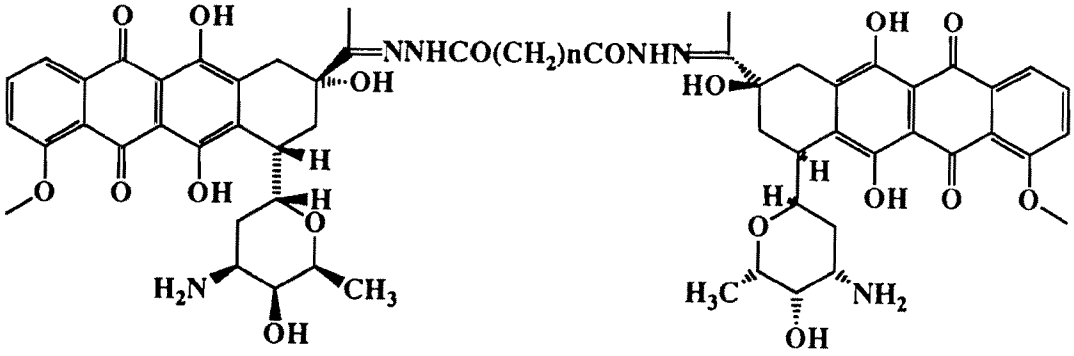


FIGURE 7 Interrelated (A) monointercalator, (B) bisintercalator, and (C) trisintercalator.



8A

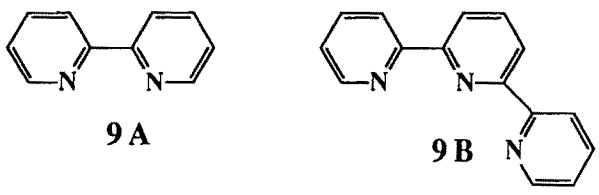


8B: n=2
8C: n=4

FIGURE 8 Daunorubicin and a dimeric daunorubicin bishydrazone.

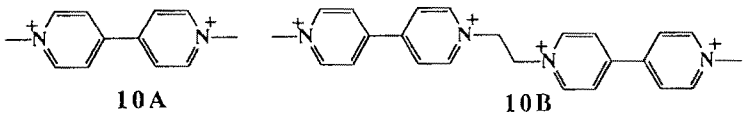
C. METAL CHELATING AGENTS

Metal chelators formed by linking pyridine units together also show a trend of increasing cytotoxicity with increase in the number of subunits linked together, so that the bipyridine **9A** in Fig. 9 inhibits L1210 leukemic cell growth with an ED_{50} of 48 μM whereas the terpyridine **9B** has an ED_{50} of only 2 μM (McFadyen *et al.*, 1985). For an example of antibacterial synergism through metal chelate formation, see Rideout (1988).



9A 9B

FIGURE 9 (A) Bipyridine and (B) terpyridine (metal chelators).



10A 10B

FIGURE 10 (A) Paraquat and (B) a paraquat dimer.

D. POLYCATIONS

Oligomerization of cations often leads to an enhancement in antimicrobial and cytotoxic activity. For example, dication **10A** in Fig. 10 inhibits B16 melanoma cells *in vitro* with an ED_{50} of 40 μM (using a thymidine incorporation assay). In contrast, the tetracation of related structure **10B** has an ED_{50} of only 4 μM in the same system (Minchin *et al.*, 1989). Polylysine can cause the permeabilization of phosphatidylcholine vesicles, a model system for permeabilization of cell membranes. A concentration of polylysine needed to cause 50% release of entrapped dye from phosphatidylcholine vesicles decreases in terms of charge ratio of polylysine to phospholipid as the molecular weight of polylysine increases. Polylysine of molecular size averaging greater than 70 KDa is twice as potent as 1.5–8 KDa polylysine (Gad *et al.*, 1982). The same trend is observed in the *in vitro* inhibition of HeLa cell growth with polylysine. The 70 KDa polylysine is 20 times as potent as 3 KDa polylysine in the inhibition of HeLa cell growth as determined on a per weight basis (Arnold *et al.*, 1979). Furthermore, the ability of 25 mg/kg of polylysine given on 5 consecutive days to extend the immediate life span of leukemia-bearing mice increases steadily with molecular weight. No antileukemic effect is observed for 3 KDa polylysine, compared with an 80% cure rate for the 70 KDa polymer (Arnold *et al.*, 1979). The polycation depicted in Fig. 11 exhibits antibacterial activity which rises dramatically with increasing molecular weight. The polymer with average molecular weight of 80,000 is more than 10,000 times as potent as the polymer of molecular weight 25,000 at a concentration of 2 μM (Ikeda *et al.*, 1986). The dicationic dequalinium (**12A**, Fig. 12) is an antineoplastic agent that selectively inhibits carcinoma cell

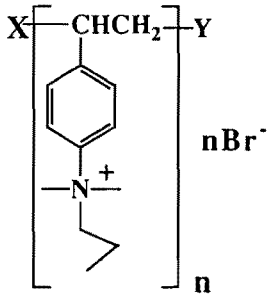


FIGURE 11 Polycations.

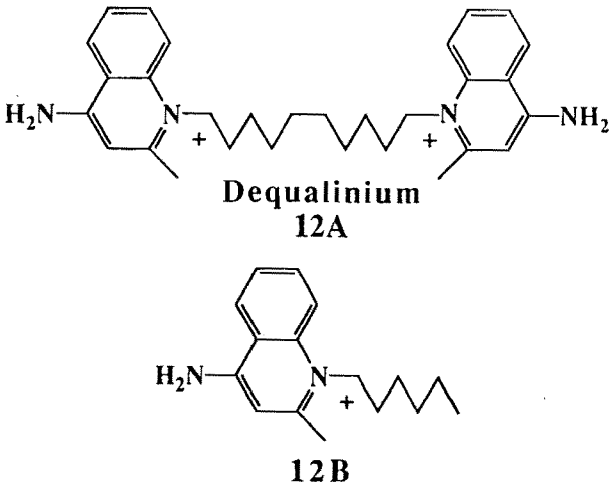


FIGURE 12 Interrelated (A) monocation and (B) dication.

growth. It is also capable of inhibiting protein kinase C with an IC_{50} less than 20 μM. In contrast, the monomeric analog **12B** fails to significantly inhibit protein kinase C even at 160 μM concentrations (Rotenberg *et al.*, 1990a). Compound **12A** is also significantly more potent than **12B** in its inhibition of MB49 murine bladder carcinoma cell growth *in vitro* (Rideout *et al.*, 1990b).

E. OTHER HOMODIMERS

Compound **13A** in Fig. 13 is an agent that binds in the minor groove of DNA and can also alkylate at that site. The monomeric **13A** has an ID_{50} of 60,000 pM against L1210 leukemia cells *in vitro*. In contrast the dimers **13B**, **13C**, and **13D** are significantly more potent with ID_{50}s of 2, 5, and 3000 pM, respectively, against L1210 under the same conditions (Mitchell *et al.*, 1989).

The dimer *bis*-ANS (**14B** in Fig. 14) binds to tubulin with a dissociation constant of 2 μM whereas the dissociation constant for ANS (**14A**) is 25 μM (Prasad *et al.*, 1986).

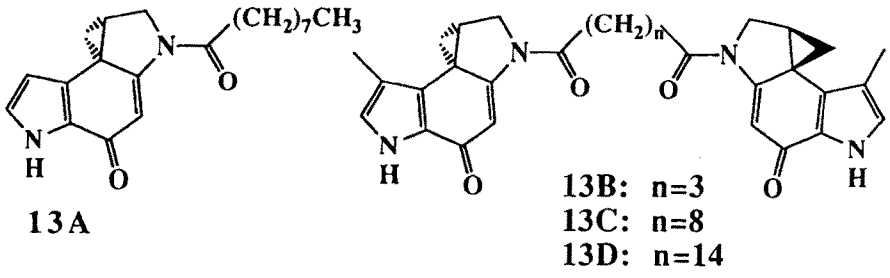


FIGURE 13 DNA alkylating agents that bind in the minor groove.

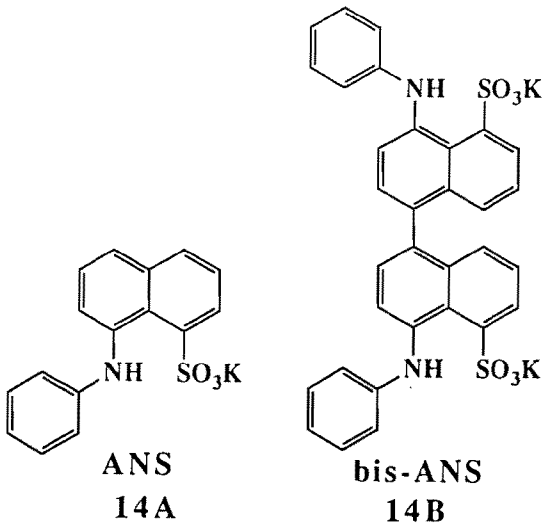


FIGURE 14 Tubulin polymerization inhibitors.

The phenomenon of enhanced antineoplastic effects upon linking of two moieties is not limited to cell killing activity. The bisamide hexamethylenebisacetamide is a substantially better inducer of erythroleukemia cell differentiation than the related monoamide *N*-methylacetamide (Reuben *et al.*, 1976).

Digallic acid (**15B** in Fig. 15) is more than 4 times as potent as the monomer gallic acid (**15A**) in terms of inhibiting the mutagenic properties of benzpyrenediolepoxide. For both molecules, anticarcinogenic activity most likely involves direct interaction with the epoxide leading to an acceleration of the rate of hydrolysis (Huang *et al.*, 1985).

F. HETERODIMERS

Linkage of two different kinds of bioactive molecules to form heterodimers can lead to dramatic increases in overall cytotoxic or antimicrobial potency.

The phosphodiester conjugate **16A** in Fig. 16 is significantly more antiviral against HIV in cell culture than either of the precursors (azidodeoxythymi-

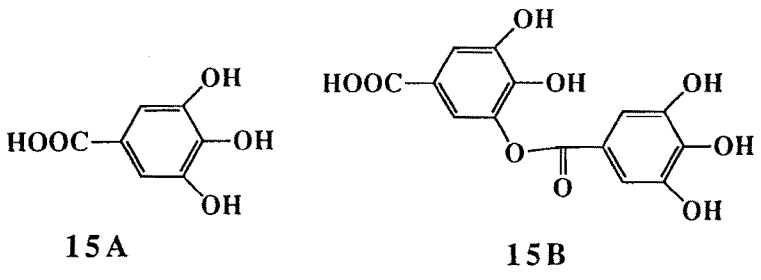


FIGURE 15 Anticarcinogens.

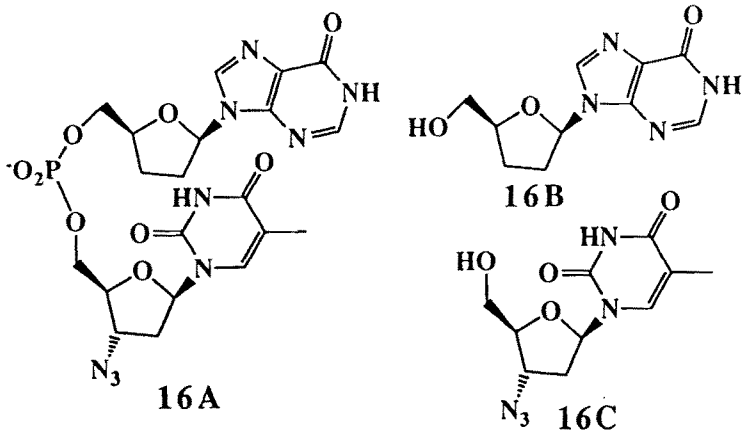
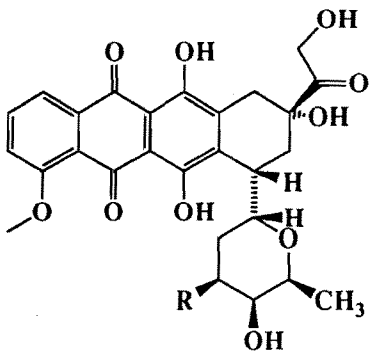


FIGURE 16 Antiretroviral agents.

dine) (**16C**) or (dideoxyinosine) (**16B**) from which it is formed. The greater activity of **16A** in comparison **16B** and **16C** is believed to be due to intracellular hydrolysis to form a mixture of **16B**, **16C**, and their monophosphates. Compounds **16B** and **16C** exhibit pronounced synergism (for further discussion, see Chapter 4 by Schinazi).

Linkage of a cyanomorpholine moiety to doxorubicin (**17A**) to form structure **17B** (Fig. 17) leads to a striking 500-fold increase of the cytotoxic potency *in vitro* against L1210 cells and a 600-fold decrease in the optimal dose for the inhibition of P388 leukemia *in vivo*. The cyanomorpholine moiety acts as an alkylating agent so that compound B is better able than A to form a covalent link to the DNA duplex after intercalation (Acton *et al.*, 1986).

For a description of synergism involving self-assembly of an amphiphilic hydrazone from a cationic hydrazine derivative and a lipophilic aldehyde, see Rideout (1986) and Rideout *et al.*, (1988), and p. 522.



17A: R=NH_2

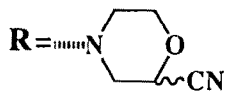
17B: R= 

FIGURE 17 (A) Doxorubicin and (B) a derivative capable of alkylating DNA.

IV. Synergism Involving Covalent Self-Assembly of Cytotoxic and Antimicrobial Agents from Less Bioactive Precursors

A self-assembling chemotherapeutic agent is a combination of relatively nontoxic prodrugs which can react with one another irreversibly *in situ* to form a more bioactive product drug (i.e., a more cytotoxic, antimicrobial, or antiviral product). In this chapter, only spontaneous nonbiochemical condensation reactions (such as hydrazone formation from a hydrazine derivative and an aldehyde or ketone) will be considered, although enzyme-catalyzed reactions might also be useful. If the velocity of the reaction between prodrugs is more rapid at the target site than in healthy tissue, then the result will be target-selective synergism (Rideout *et al.*, 1990). The target site could be a tumor cell, a tumor extracellular space, a pathogenic microbe, a virally infected cell, or a subcellular compartment in which the pathogenic microbe resides. The resulting chemotherapeutic effect will be significantly enhanced through selective self-assembly at the target site, while the toxic effects of the drug on healthy tissue (where the velocity of drug formation is lower) will be enhanced only slightly. The combination of prodrugs will be more selective (although less potent) than the preformed product drug because of the unique advantages imparted through bimolecular kinetics. At optimized dose levels, the combination would be capable of providing more therapeutic benefits with fewer side effects than the performed product. The combination will be both more selective and more potent than either prodrug alone.

Figure 18 depicts one way in which the self-assembling drug approach could be used to amplify selectivity. First target and normal cells are exposed to identical concentrations of nontoxic prodrugs X and Y. Then X and Y are taken up by the target cell with slight distribution selectivity. As a result, let us suppose that their concentrations become 3-fold higher in the target than in normal tissues. Finally, X and Y react to form the cytotoxic product X–Y. Because the velocity of X–Y formation is proportional to the product of the concentrations of X and Y, the resulting concentration of cytotoxin X–Y will initially be 9-fold higher in the target. This analysis assumes identical second-order rate constants in both cells. Because of the multiplicative nature of second-order reaction kinetics (i.e., $d[X–Y]/dt = k_2[X][Y]$, where k_2 is the second-order rate constant), the resulting distribution selectivity of X–Y becomes amplified relative to the selectivities of X or Y alone. This 9-fold distribution selectivity will be independent of any selectivity inherent in X–Y as a preformed drug. If X–Y exhibits any selectivity due to distribution properties and/or innate biochemical or cytological differences between target and normal cells, the overall selectivity of target inhibition by combinations of X and Y will ultimately exceed 9. Although the kinetic analysis in this example is simplified, more sophisticated computer simulations which take into

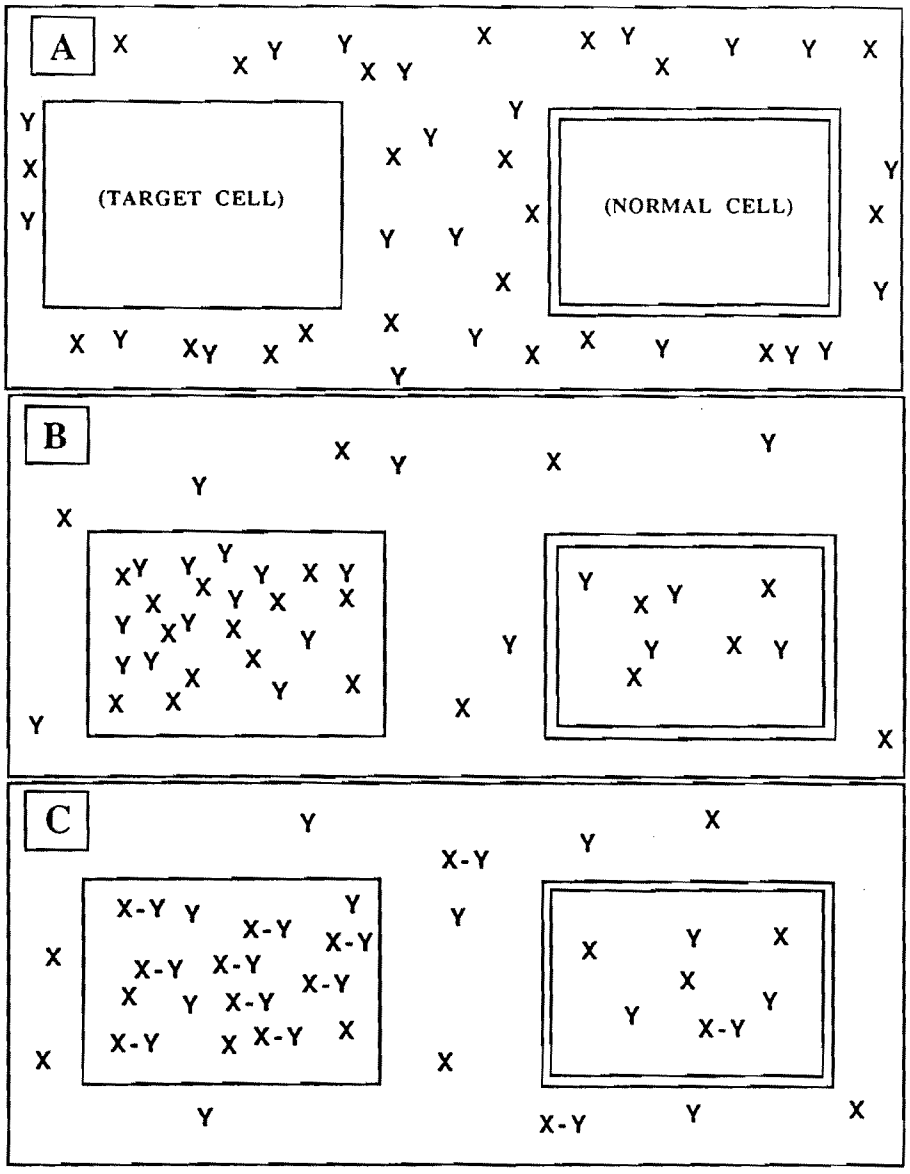


FIGURE 18 The enhancement of distribution selectivity effects using self assembly. Reprinted from Rideout *et al.*, 1990a by permission. Copyright © 1990, John Wiley & Sons, Inc.

(A) Two relatively nontoxic precursors (prodrugs) X and Y are administered.

(B) These accumulate *somewhat* selectively in the target cell.

(C) X and Y combine to form the more cytotoxic (or more antimicrobial) drug X–Y *more* selectively in the target cell because of the second order rate law.

account the simultaneous nature of product formation, prodrug and product efflux, and prodrug and product influx also predict that selectivity amplification should be possible using this approach (Rideout and Rideout, 1991). A possible example of this phenomenon, involving combinations of a triarylalkylphosphonium-derived aldehyde and an acylhydrazide (Rideout *et al.*, 1990), is described on p. 523.

Figure 18 represents a cell-targeting approach. An equally valid approach involves targeting the environment *near* the tumor cell, pathogenic microbe,

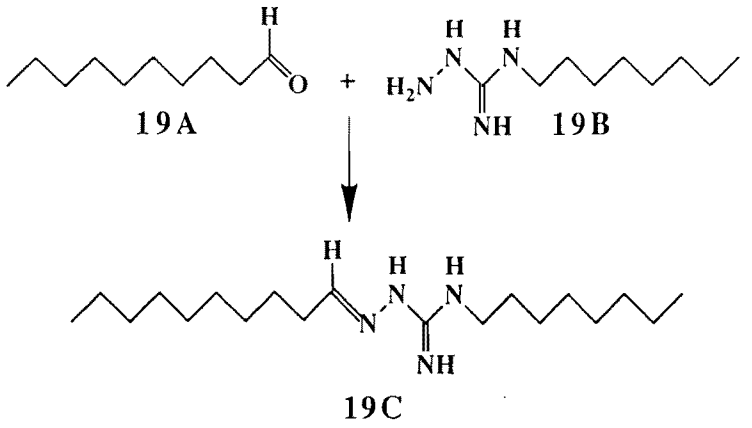


FIGURE 19 A self-assembling amphiphilic cation.

virus, or virally infected cell (Rideout *et al.*, 1988). Hematoporphyrin derivative (fotofrin II) and lipiodol are among the agents which tend to accumulate in the extracellular environment around tumor cells in solid tumors (Bohmer and Morstyn, 1985; Ogita *et al.*, 1987). An example of environment-selective synergism involving an *in vitro* model system has been described: a combination of **19A** and **19B** (Fig. 19) exhibits selective cytotoxic synergism in a low-serum environment by virtue of more rapid formation of hydrazone **19C** (Rideout *et al.*, 1988).

Another approach to target-selective synergism using self-assembling combinations involves the exploitation of differences in the second-order rate constant k_2 for the reactions in the target compared with normal tissues. If k_2 in the target is larger than k_2 in the normal tissue, then selective formation of bioactive product from prodrugs would be more rapid in the target even for prodrugs which could not accumulate selectively in the target. This form of selectivity, unique to self-assembling chemotherapeutic agents, is called assembly-rate-constant selectivity (Rideout *et al.*, 1988). Examples are described in Sections IVC and IVD.

A. BIOACTIVITY OF HYDRAZONES

Hydrazones can exhibit chemotherapeutic activity clinically, in animals, and/or *in vitro* through a very wide variety of mechanisms, including DNA intercalation, cell membrane lysis, inhibition of bacterial cell wall synthesis, reverse transcriptase inhibition, inhibition of tubulin aggregation, and so forth (reviewed by Rideout *et al.*, 1990a). In addition, viruses can be inhibited by a hydrazone that inhibits ribonucleotide reductase (see Chapter 8 by Spector and Fyfe), the antineoplastic hydrazone antibiotic luzopeptin A can form interduplex DNA cross-links (Searle *et al.*, 1989), the anticoccidial hydrazone robenzidine inhibits oxidative phosphorylation in mammalian liver cells (Wong *et al.*, 1972), several thiosemicarbazones of 2-acetylpyridine show

activity *in vivo* against mice bearing the malarial parasite *Plasmodium berghei* (Klayman *et al.*, 1979), and antimitotic hydrazones have been described (Temple, 1990).

B. ANTINEOPLASTIC COMBINATIONS OF ALDEHYDES AND HYDRAZINE DERIVATIVES

We have developed a self-assembling protein kinase C inhibitor consisting of aldehyde **20A** and acylhydrazide **20B**. Testing of PKC inhibition by this combination has been carried out in collaboration with S. Rotenberg and I. B. Weinstein (Columbia University Cancer Center, New York). The adduct formed from **20A** and **20B** is hydrazone **20C**, a dication that inhibits protein kinase C activity with an IC_{50} value of 20.4 μM. In contrast, the values for **20A** and **20B** are both >200 mM. Inhibition of α2-PKC by **20A** alone, **20B** alone, or by **20C** alone shows no significant time dependence. However, when α2-PKC is exposed to 200 μM **20A** and 200 μM **20B** in combination, the activity of the enzyme drops from 48% of control at 0 min to 15% of control at 70 min. The percentage of control values are 87 and 40, respectively, for 100 μM + 100 μM. Kinetic studies using HPLC demonstrate the formation of approximately 27 μM **20C** from 200 μM **20A** + 200 μM **20B** under the PKC assay conditions after 60 min. In conclusion, the combination of **20A** and **20B** exhibits synergistic inhibition of PKC in a time-dependent manner through direct assembly to form the more potent inhibitor **20C** (Rotenberg *et al.*, 1990b).

The structures of phosphonium salts **20A** and **20B** resemble tetraphenylphosphonium, a delocalized lipophilic cation that accumulates selectively in many carcinoma cells relative to most untransformed cells (see Rideout *et al.*, 1989, and Chen, 1988). Tetraphenylphosphonium and **20B** all inhibit Ehrlich–Lettre ascites (ELA), PaCa-2 human pancreatic carcinoma, and MB49 murine bladder carcinoma selectively relative to CV-1 untransformed monkey kidney cells, whereas cisplatin and cytarabine are unselective (Rideout *et al.*, 1989, 1990 unpublished observations). We predicted that **20A** and **20B** would accumulate selectively in carcinoma cells, combining selectively therein to form **20C** (as in Fig. 20). Perhaps because it is a better PKC inhibitor, **20C** is more cytotoxic than either **20A** or **20B**. (Rideout *et al.*, 1990). Carcinoma-selective accumulation of **20A** and **20B** should lead to carcinoma-selective formation of the more cytotoxic **20C**, and thus to carcinoma-selective synergism between **20A** and **20B**. As predicted, combinations of **20A** and **20B** exhibit synergism in their inhibition of ELA cells, but not in their inhibition of CV-1 cells, as determined by isobologram and combination index methodologies (Rideout *et al.*, 1990). Combinations related to **20A** + **20B** that cannot form hydrazones (e.g., **20A** + **20D** and **20B** + **20E**) do *not* exhibit synergism in their inhibition of ELA cell growth, consistent with the hypothesis that hydrazone formation is the mechanism of synergism

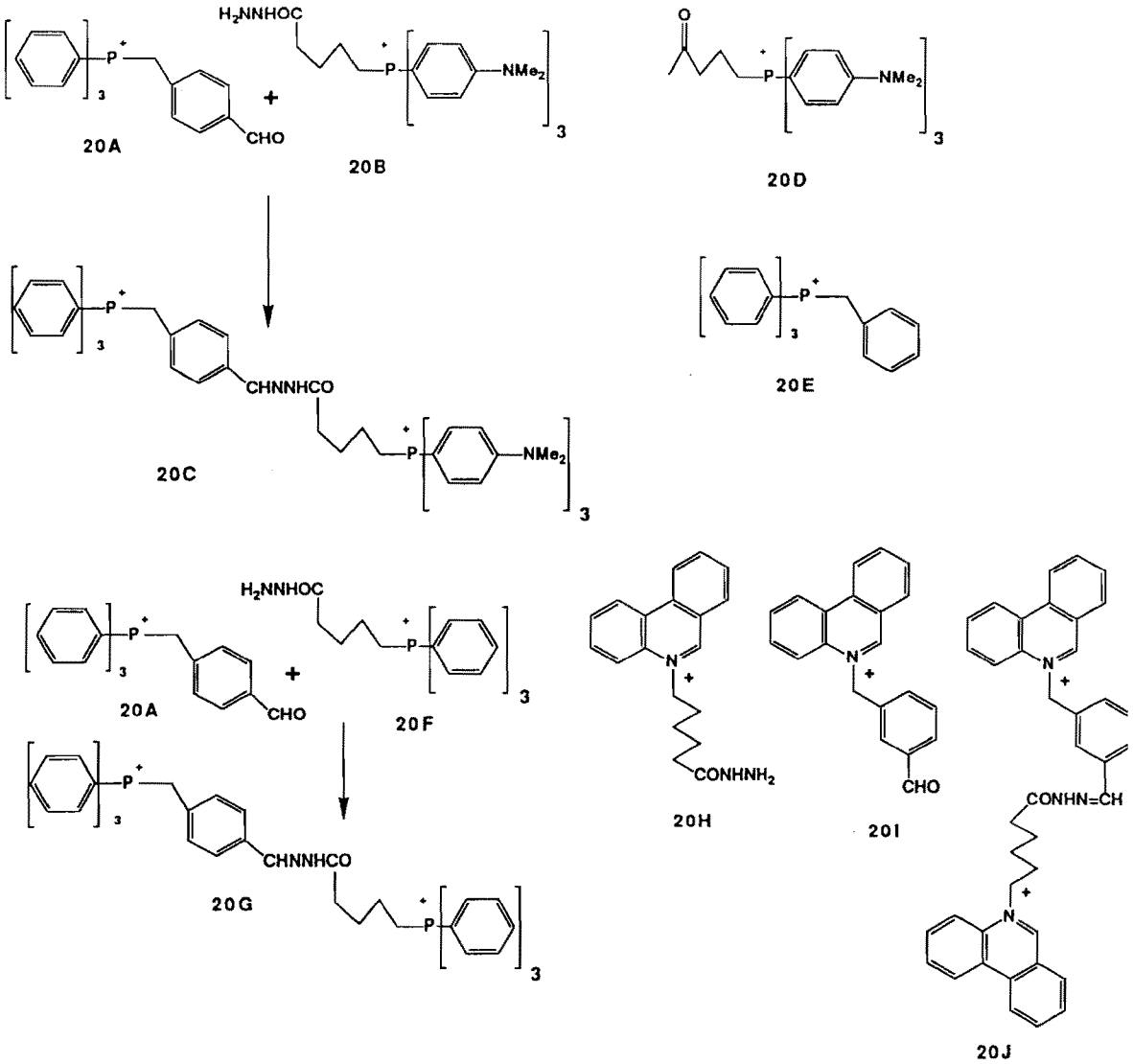


FIGURE 20 Synergistic antineoplastic combinations of delocalized lipophilic cations.

between **20A** and **20B**. In summary, ELA cells probably selectively catalyze hydrazone **20C** formation from **20A** and **20B** by concentrating the two together in the cytoplasm and/or mitochondria. The **20C** thus formed inhibits cell growth more effectively than the precursors (Rideout *et al.*, 1990).

Aldehyde **20A** and acylhydrazine **20F** are both less cytostatic than their hydrazone **20G** in assays using PaCa-2 (2-day exposure) and CV-1 cells (2-day exposure). The cytostatic synergism between **20A** and **20F** against PaCa-2 carcinoma cells (2-day assay, isobologram and combination index analysis) is most likely due to the formation of the more cytostatic hydrazone **20G**. Modest carcinoma-selective synergism against PaCa-2 cells has also been observed for combinations of **20A** and **20F**. In other words, a combination of 212 μg/ml of **20F** with 31 μg/ml of **20A** is more selective than either

424 μg/ml of **20F** alone or 62 μg/ml of **20A** alone (Rideout *et al.*, unpublished observations).

A combination of **20H** and **20I** is quite synergistic against PaCa-2 carcinoma cells (2-day exposure). Assuming mutually exclusive mechanism, at a 50% inhibition level the combination indexes (see Chapter 2 by Chou) are less than 0.488 for **20H** + **20I** at a 10.2:1 molar ratio, and less than 0.568 at a 4.4:1 ratio. At a 1:1.62 molar ratio, the combination **20H** + **201** is also synergistic against MB49 cells, with a combination index less than 0.68 (2-day exposure, 50% effect level). The synergism observed in this combination probably involves the *in situ* formation of the dicationic hydrazone **20J** (Rideout *et al.*, unpublished observations).

C. ANTIMICROBIAL COMBINATIONS OF ALDEHYDES AND HYDRAZINE DERIVATIVES

Mixtures of aldehydes and acylhydrazines that can react *in situ* to form antimicrobial hydrazones demonstrated greater degrees of synergism against the intracellular pathogen *Salmonella typhimurium* at pH 5 than at pH 7.4. These mixtures include 5-nitro-2-furaldehyde (**21D**) plus semicarbazide (**21E**) and 2-hydrazinopyridine (**21A**) plus pyridine-2-carboxaldehyde (**21B**) (Fig. 21). Combinations are more selectively toxic to bacteria at pH 5 (versus pH 7.4) than individual precursors and preformed hydrazone products because acid-catalyzed hydrazone bond formation plays a role only for the combination (Rideout *et al.*, 1988).

The precursors **21D** and **21E** are at least 100 times as soluble in water as their product **21F**. This suggests that self-assembling combinations of soluble prodrugs might be used as a means of administering drugs that are otherwise too insoluble to be used as single agents in preassembled form.

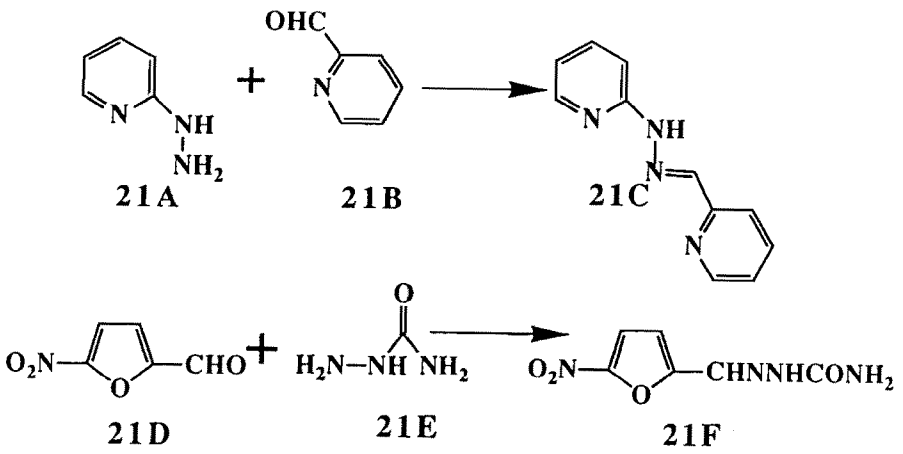


FIGURE 21 Synergistic antibacterial combinations.

Pyridoxal and isoniazid exhibit antimalarial synergism due to the formation of an iron-chelating hydrazone *in situ* (see Chapter 5 by Vennerstron *et al.*).

D. HYDRAZONE FORMATION IN MACROPHAGES: PROGRESS TOWARD SELF-ASSEMBLING ANTIRETROVIRAL AGENTS

Hydrazine **22A** (Fig. 22) accumulates with some selectivity in macrophages through rapid pinocytic accumulation in lysosomes (Swanson *et al.*, 1985). This fact suggests that hydrazones might be found selectively in macrophages from **22A** and various aldehydes. If such a hydrazone possesses antiretroviral activity, the corresponding aldehyde and acylhydrazide could be used to selectively target the hydrazone to macrophages which act as reservior for the AIDS virus (Fauci, 1988; Pauza, 1988).

Hydrazones **22B, 22C**, and **22D**, all derived from hydrazine of **22A**, inhibit reverse transcriptase isolated from HIV-1 and/or feline immunodeficiency virus (FIV) (Rideout *et al.*, unpublished observations). FIV is a lentivirus that

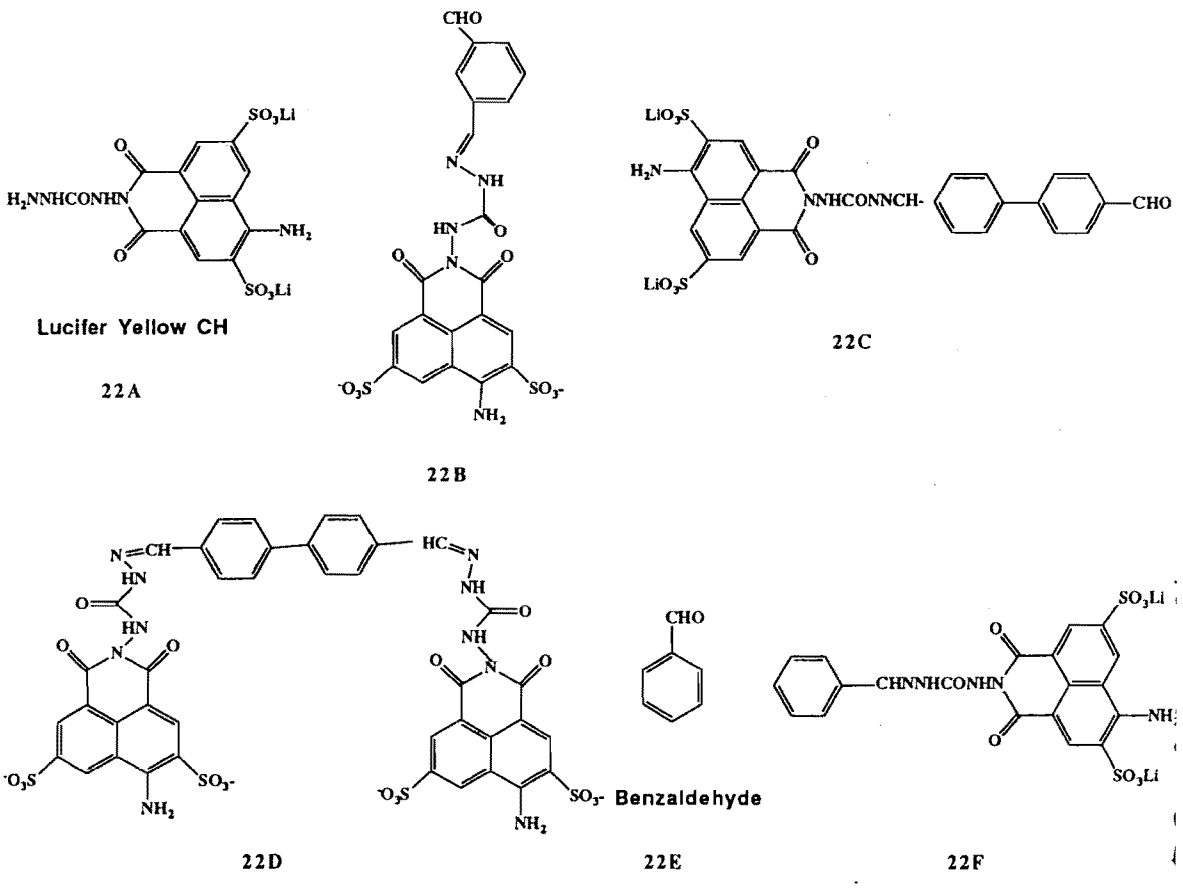


FIGURE 22 (A) Lucifer Yellow CH and (B–D,F) antiretroviral hydrazones.

causes a syndrome resembling AIDS in cats (Pedersen *et al.*, 1987; Talbott *et al.*, 1989). Hydrazone **22C** can inhibit FIV proliferation by >99% at nontoxic doses (Rideout *et al.*, unpublished observations). Hydrazine **22A** has very low cytotoxicity (Swanson *et al.*, 1985) and whole animal toxicity (S. C. Silverstein, unpublished observations) but has no measurable reverse transcriptase-inhibiting or antiretroviral activity (Swanson *et al.*, 1985). Together these results suggest that it may be possible to achieve macrophage-selective delivery of antiretroviral hydrazones related to **22C** by administering them as a combination of less toxic aldehyde and acylhydrazide prodrugs (which need not have antiviral activity as single agents in order to be effective as prodrugs).

Hydrazone **22F** forms from benzaldehyde (**22E**) and the hydrazine derivative Lucifer Yellow CH (**22A**) at 37°C with a rate constant that is approximately 100 times higher at pH 5.0 than at pH 7.4 (Rideout *et al.*, unpublished observations). The hydrazine **22A** is taken up by macrophages through pinocytosis (Swanson *et al.*, 1985) and concentrated in lysosomes, in which the pH is close to 5 (Roos and Boron, 1981). When TPA-pretreated U937 human leukemic cells are treated for 24 hr with **22A** (2 mg/ml), washed with PBS, and treated with 471 μM benzaldehyde (S3B) in PBS + 8% serum for 20 hr at pH 7.4, half of the **22A** is converted to hydrazone **22F** [TPA-treated U937 cells are a convenient model for human monocyte/macrophages (Harris and Ralph, 1985)]. If the cells are killed by hypoosmotic shock and freezing just prior to **22A** addition, less than 10% of **22A** is converted to **22F**. Addition of chloroquine, which lowers the lysosomal pH, substantially reduces the rate of hydrazone formation in the cell extracts. If human monocyte/macrophages are treated with **22A** for 24 hr, and **22E** is then added without washing away external **22A**, **22F** forms with a $t_{1/2}$ of 2 hr in the cell extracts. In contrast, there is less than 10% conversion to **22F** in the extracellular medium (pH 7.4) after 20 hr. These data are consistent with accelerated hydrazone formation inside the cell lysosomes through pinocytosis of **22A**, followed by diffusion of **22E** into the lysosomes, and acid catalysis of **22F** formation (Rideout *et al.*, unpublished observations).

The reaction between **22A** and **22E** can also occur inside living mice. BALB/c female mice were treated ip with thioglycolate broth to induce macrophages (Campbell, 1984). After 4 days, they were treated with **22A** (120 mg/kg ip). After 24 hr, **22E** (30 mg/kg) was added. After 2 more hr, ip macrophages and extracellular fluids were isolated postsacrifice and analyzed using fluorescence-linked HPLC. The hydrazone **22F** was detected (using HPLC) inside the macrophages and in the extracellular fluid (Rideout *et al.*, unpublished observations). This result is important because it suggests that antiretrovial hydrazones could be delivered to macrophages by aldehyde and acylhydrazide administration *in vivo*. Perhaps more important, the experiment demonstrates that a hydrazone can be formed within a live animal from an aldehyde and a hydrazine derivative, even when *both* of these precursors are administered rather than being endogenous metabolites.

In summary, selective accumulation of one or both prodrug precursors in or near the target and/or an enhanced reactivity between the prodrug precursors in the target could be used to attain target-selective synergism and amplified selective inhibition of the target. The key strength of the approach is flexibility. Useful drug self-assembly could occur inside target cells, in the extracellular environment associated with the target, or in a particular type of subcellular compartment containing a target pathogen. It could occur in solution, in membranes, on proteins, or on nucleic acids. The concept is neither restricted to any one type of bimolecular reaction, nor to any one mechanism of selective accumulation, nor to any type of environmental difference responsible for assembly-rate-constant selectivity, nor to any particular mechanism of target inhibition by the product drug.

V. Antagonism Involving Covalent Self-Assembly

One strategy for enhancing the therapeutic index of a chemotherapeutic agent involves decreasing the host toxicity through selective chemical combination with a second agent in host tissue, forming a third, less-toxic product. Most of the reported examples of this covalent modulation strategy involve combinations of cisplatinum and ligands. Examples of antagonism involving iron chelation and epoxide hydrolysis will also be discussed here.

Chelators and thiols can protect mammals against the toxicity of metal ions. For example, *N*-acetylcysteine can facilitate the excretion of lead and cadmium from rats. The chelator "tiron" can protect against the toxicity of uranium oxide ions in Swiss mice and the clinically used chelating agent dimercaprol can protect against acute cadmium toxicity in rats (Ottenwälder and Simon, 1987; Domingo *et al.*, 1989; Peele *et al.*, 1988). More interesting from the point of view of chemotherapy is the reduction of the toxicity of platinum-derived antitumor agents such as cisplatin and carboplatin by a variety of nucleophilic ligands, mostly thiols (see next section).

A. CISPLATIN MODULATION WITH CHLORIDE ANION

Much of the toxicity of the anticancer agent cisplatin to human beings is believed to be due to the reaction of hydrated forms of the drug such as $Pt(NH_3)_2(H_2O)(OH)^+$ with proteins (Dedon and Borch, 1987). One of the key dose-limiting toxicities of cisplatin involves damage to kidneys. The hydrated form of cisplatin inactivates the rat renal proximal tubular enzyme

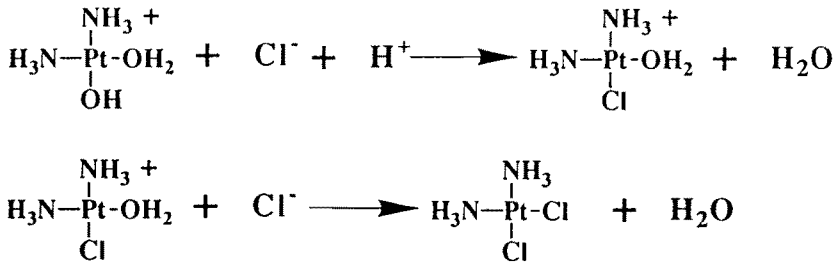


FIGURE 23 Detoxification of nephrotoxic cisplatin metabolites through self-assembly with chloride ion.

γ-glutamyltranspeptidase (γ-GT) at 2 μM with a half-life of just over 1 hr, wheras cisplatin itself in water requires over 3 hr at the same concentration (Dedon and Borch, 1987). In the presence of 100 mM sodium chloride, cisplatin requires more than 5 hr to inactivate γ-GT by 50% under the same conditions. This is believed to be due to a self-assembly reaction between chloride ion and various toxic hydrated cisplatin species as shown in Fig. 23 (Dedon and Borch, 1987). Chloride ion in the form of hypertonic saline is used to decrease the kidney toxicity of cisplatin in the clinic through this self-assembly reaction (Fuks *et al.*, 1987; Aamdal *et al.*, 1987). Related studies of cisplatin hydration and reformation include those by Corden (1987) and by LeRoy and Thompson (1989).

B. CISPLATIN MODULATION WITH THIOLS AND SELENIUM

A number of sulfur nucleophiles which form complexes with platinum derivatives involving sulfur–platinum bonds have been shown to reverse many of the toxic side effects of cisplatin and related platinum-containing anticancer agents. The chemical structures of several such nucleophiles are shown in Fig. 24. The modulation of cisplatin toxicity by thiols was recently reviewed by Leyland-Jones (1988).

1. Thiosulfate

In a classic study, Howell *et al.* (1983) showed that thiosulfate delivered intravenously can protect human subjects against the renal toxicity of cisplatin administered intraperitoneally. They showed that unreacted cisplatin could be found in high levels in plasma, despite the fact that concentrations of thiosulfate present in the plasma were 280 to 950 times higher than plasma cisplatin concentrations. It was proposed that thiosulfate becomes extensively concentrated in the kidneys, producing a high enough local concentration to

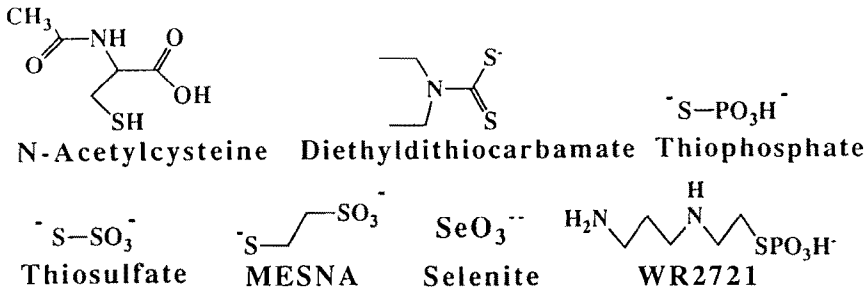


FIGURE 24 Sulfur nucleophiles for cisplatin and alkylating agent "rescue" (antagonism) involving Pt-S, Pt-Se, or C-S bond formation.

neutralize cisplatin and its metabolites in renal tubules without compromising the antineoplastic activity of cisplatin elsewhere. In other words, kidney-selective detoxification of cisplatin occurs through kidney selective assembly between thiosulfate and cisplatin. In a later study, Goel *et al.* (1989) showed that sodium thiosulfate can also prevent peripheral neuropathy caused by cisplatin and perhaps even decrease some of the central nervous system effects such as fatigue and vomiting that can be associated with clinical cisplatin therapy. Uozumi and Litterst (1986) showed that although thiosulfate protects against kidney damage by cisplatin, thiosulfate does not induce a significant change in the subcellular distribution of platinum within rat kidney cells. The authors concluded that thiosulfate acts by forming a nontoxic complex with cisplatin in the kidney rather than influencing the total amount of platinum metal that reaches vulnerable targets within kidney cells. Thiosulfate was shown to protect guinea pigs from hearing loss induced by cisplatin. This observation is significant because hearing loss is one of the irreversible side effects which is dose limiting in cisplatin usage (Otto *et al.*, 1988).

Cisplatin has been shown to react with 2 m*M* solutions of thiosulfate, being completely consumed within a few hours at 37°C and forming convalent adducts in aqueous buffers (Elferink *et al.*, 1986). Carboplatin is considerably less reactive under the same conditions. Electron opaque bodies are found in the lysosomes of kidney tubule cells when cisplatin and thiosulfate are administered together to male Wistar rats, but not when either cisplatin or thiosulfate is administered alone. On microanalysis these opaque bodies have been shown to contain high levels of sulfur and platinum, suggesting that covalent combination between thiosulfate (or its metabolites) and platinum-containing species had occurred *in vivo* (Berry and Lespinats, 1988). The reaction between thiosulfate and cisplatin has been shown by HPLC to occur at a rate of about 0.06 mol/sec at 37°C (Dedon and Borch, 1987). Thiosulfate is incapable of reacting with cisplatin/thioprotein adducts and thus cannot reverse damage caused by cisplatin to proteins such as those in the renal tubules. As a result, thiosulfate must be given prior to and simultaneous with cisplatin in order to decrease the toxic effects of the drug (Dedon and Borch,

1987; Elferink *et al.*, 1986.) In addition, thiosulfate does reduce the antitumor activity of cisplatin in animals when certain procedures are used (Aamdal *et al.*, 1988).

2. Diethyldithiocarbamate

In contrast to thiosulfate, diethyldithiocarbamate (DDTC) is capable of reversing adducts between cisplatin and cysteine-containing proteins, thereby freeing the protein. As a result, DDTC can be administered after cisplatin and still significantly reduce the host toxicity of the heavy metal complex (Basinger *et al.*, 1989; Dedon and Borch, 1987).

Diethyldithiocarbamate (DDTC) is unusual because it can reverse cisplatin/thiol protein adducts. For example, DDTC reactivates γ-glutamyltranspeptidase (γ-GT) which can become inhibited due to cisplatin derivitization (γ-GT is an important enzyme found at high levels in kidney tissue). On the other hand, DDTC is not able to reverse the cross-links which cisplatin introduces between adjacent guanine residues in DNA. In other words, DDTC can reverse the nephrotoxic lesions of cisplatin while sparing the cell-killing, potentially antineoplastic lesions of cisplatin (Bodenner *et al.*, 1986). Unfortunately, at concentrations which are sufficient to reverse the toxic effects of cisplatin, DDTC itself exhibits quite significant toxicity (Leyland-Jones, 1988).

DDTC is also capable of reversing adducts between cisplatin and human α2-macroglobulin (Gonias *et al.*, 1984). It has been shown to protect animals from the kidney and bone marrow toxicity of cisplatin at levels that do not compromise the antineoplastic activity of cisplatin (Gonias *et al.*, 1984; Schmalbach and Borch, 1989). It also suppresses the bone marrow toxicity of the cisplatin analog, carboplatin (Schmalbach and Borch, 1989).

3. Other Sulfur Nucleophiles and Selenium

The radioprotectant *S*-2-(3-aminopropylamino)-ethylphosphorothioic acid (WR2721) is capable of protecting peripheral nerves from cisplatin toxicity in human subjects (Mollman *et al.*, 1988). Glover *et al.*, (1984) showed in a clinical trial that the organic thiophosphate WR2721 provides some protection against cisplatin-mediated nephrotoxicity in human subjects. Presumably, WR2721 is dephosphonylated to the thiol *in vivo*. More recent clinical trials of WR2721/cisplatin are encouraging (Glover *et al.*, 1989; Mahaney, 1990). The thiol 2-mercaptoethanesulfonate (MESNA) not only protects rats against the nephrotoxicity of cisplatin, but also completely suppresses cisplatin induced benign and malignant neoplasm formation in these animals. On the other hand, it does not seem to have an effect on the antineoplastic effects of cisplatin against L1210 leukemia in mice (Kempf and Ivankovic, 1987). Selenium in the form of sodium selenite dramatically reduces the intestinal

and kidney toxicity and associated lethality of cisplatin (Satoh *et al.*, 1989). Electron microprobe and metal distribution studies suggest that direct selenium–platinum bond formation is involved in the detoxification (Satoh *et al.*, 1989; Naganuma *et al.*, 1983; Berry and Lespinats, 1988). Selenium (in the form of sodium selenate) has also been shown to decrease cisplatin toxicity. A direct combination mechanism has been proposed (Ohkawa *et al.*, 1988).

One must be cautious in interpreting many of the results as involving simple direct combination between cisplatin and the antagonizing agent. It is possible in some instances to induce the synthesis of biological thiols such as metallothionein which is induced by bismuth salts (Satoh *et al.*, 1988). It is possible that the administration of selenium or of thiols such as thiosulfate causes the induction of endogenous thiol synthesis or of enhanced levels of enzymes such as glutathione-*S*-transferase which can catalyze the detoxification of agents such as cisplatin. Cisplatin reacts with 5 μM (excess) glutathione to form a product with a sulfur–platinum bond. The reaction half life is 200 min at 40°C (Corden, 1987). There is still only limited direct evidence involving animal studies for significant direct bond information between cisplatinum and thiols or selenium administered exogenously.

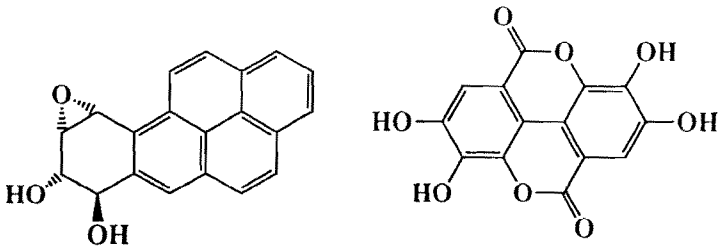
C. IRON CHELATION

8-hydroxyquinoline and a one-to-one 8-hydroxyquinoline:Fe(II) complex exhibit antibacterial antagonism by virtue of self-assembly to form 2(8-hydroxyquinoline):Fe(II), which is much less active that the 1:1 complex. At physiological iron concentrations, 8-hydroxyquinoline is active as an antibacterial agent at 10 μM yet much *less* active at 1000 μM concentrations, an unusual phenomenon known as "concentration quenching" (see Chapter 5 by Vennerstrom *et al.*).

D. EPOXIDE HYDROLYSIS

Plant-derived phenols such as tannic and ellagic acid are part of a large and diverse group of compounds that can prevent the mammalian cell mutagenesis and carcinogenesis by certain chemical agents and radiation (Wattenberg, 1985; Mukhtar *et al.*, 1988). Tannic acid is particularly active in preventing the induction of skin tumorigenesis in mice by the carcinogens dimethylbenzanthracene, benzpyrene, and 3-methylcholanthrene. By comparing a large number of plant flavonoids, Huang *et al.* (1983) have shown that phenolic groups are essential for the inhibition of mutagenicity by bay region diolepoxides.

Ellagic acid (**25B** in Fig. 25) is a naturally occurring plant phenol that is normally ingested by humans. A 10 μM concentration of **25B** increases the



Benzpyrenediolepoxide **Ellagic Acid**

FIGURE 25 A carcinogen (benzpyrenediolepoxide) and an anticarcinogen (ellagic acid).

rate of disappearance of the carcinogen benzpyrene-7,8-diol-9,10-epoxide (**25A**) by a factor of 20 at pH7. At 2 μM, **25B** can inhibit the mutagenicity of 0.1 μM **25A** by 50% in the Ames *Salmonella typhimurium* mutagenicity assay (Wood *et al.*, 1982). Despite the simplicity of elagic acid's structure, the rate of the reaction between **25B** and **25A** has a surprisingly high second-order rate constant of 560 molar^{-1} sec^{-1}. The toxic antagonism of **25A**, a metabolite of the ubiquitous carcinogen benzpyrene, by a variety of phenols found in common foods is of possible importance in the prevention of cancer through control of diet. Tannic acid inhibits the mutagenicity of **25A** against *S. typhimurium* by 50% at 1 μM concentrations (Huang *et al.*, 1985).

The rates of hydrolysis of **25A** by a variety of soluble aromatic compounds increase in the order ferullic acid, caffeic acid, chlorogenic acid, flavin mononucleotide, and elagic acid (Wood *et al.*, 1982). The ability of these compounds to protect V79 Chinese hamster cells or *S. typhimurium* cells against mutagenesis increases in the same order, suggesting that in cell culture as well as *in vitro* direct catalysis of **25A** hydrolysis is the mechanism of protection for these compounds. In other words, these aromatic compounds inhibit the mutagenicity of **25A** through a direct interaction (Wood *et al.*, 1982).

E. MODULATION OF ALKYLATING AGENTS WITH THIOLS

The thiol 2-mercaptoethane sulfonate sodium (MESNA) is known to react with toxic metabolites of cyclophosphamide such as acrolein, forming a carbon–sulfur bond. MESNA greatly diminishes the toxicity of these metabolites, which probably do not contribute the antineoplastic effects of cyclophosphamide. MESNA is now being used clinically in an attempt to decrease the kidney toxicity of alkylating agents such as ifosfamide (Goren *et al.*, 1989). Similarly, glutathione monoethyl ester can selectively protect the liver from high doses of alkylating agents such cyclophosphamide or BCNU without compromising anticancer activity against fibrosarcoma in mice (Teicher *et al.*, 1988). Like MESNA, *N*-acetylcysteine can protect against the toxicity of

phosphoramide mustard without compromising its cytotoxic activity. It was shown that although *N*-acetylcysteine can react with acolein, an undesirable toxic metabolite of cyclophosphamide, *N*-acetylcysteine does *not* react with phosphoramide mustard, the active antineoplastic metabolite of cyclophosphomide (Seitz *et al.*, 1989).

In summary, chemotherapeutic synergism and antagonism between two agents can be mediated through direct chemical reaction between the agents, creating a covalent bond and forming a new molecule or molecules. Some of the best characterized examples of this very straight forward mechanism of interaction (covalent modulation) involve carbon–nitrogen double bond or platinum sulfur bond formation. Other types of bimolecular reactions that can be rapid under near-physiological conditions (e.g. Diels–Alder condensations) might someday be applied in novel synergistic or antagonistic combinations. Enhancement of the therapeutic index can be achieved using covalent modulation if reactions leading to a more active product occur more rapidly at the target site or if reactions producing a less toxic product occur more rapidly in normal tissues.

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