CANCER CHEMOTHERAPY[1,2]

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The development of chemotherapeutic agents for use against malignant diseases in man poses one of the great challenges to medical science, for unlike infectious diseases, where successful chemotherapy can be applied in many instances, effective drug therapy of neoplasia is the exception. The relatively unsuccessful chemotherapeutic approach to cancer relates in part to the fact that biochemical differences between the tissues of the host and the neoplastic cell are slight. In addition, in contrast to antimicrobial chemotherapy, the contribution of host defence mechanisms to therapy of cancer appears to be minimal or absent. The cancer chemotherapist must, therefore, rely upon quantitative differences in the metabolic makeup of tumor and normal cells, as well as upon differences between cell types in the duration of the reproductive cycle; these factors determine the relatively low therapeutic indices of antineoplastic agents. Nevertheless, there is now available a large array of chemical agents in the therapeutic armamentarium capable of interfering with many of the metabolic processes required for the growth and survival of mammalian cells. Certain of these drugs have some utility in the treatment of malignant diseases of man; these agents, as well as those compounds which appear to be effective only against experimental tumors in animals, might be employed to greater advantage in clinical situations if complete information were available as to the factors, both pharmacological and biochemical, that are involved in their mode of action. Such knowledge would aid in (*a*) the development of diagnostic tests for sensitivity of neoplastic populations; (*b*) the selection of treatment regimens; and (*c*) the rational choice of other agents to employ in combination in an effort to achieve synergistic antineoplastic potential. Accordingly, we have chosen to review the chemotherapy of cancer primarily in terms of new information on the mechanism of action of selected tumor-inhibitory drugs that have demonstrated clinical utility, as well as some new developments in cancer chemotherapy that show promise for the future.

[1] The survey of literature pertaining to this review was concluded in July 1968.

[2] Abbreviations used in this review are: ara-C (arabinosyl cytosine); ara-CTP (arabinosyl cytosine triphosphate); ara-U (arabinosyl uracil); KTS [kethoxal *bis* (thiosemicarbazone)]; MIH [N-isopropyl-α-(2-methylhydrazino)-*p*-toluamide]; 6-MMPR (6-methylthiopurine ribonucleoside); 6-MP (6-mercaptopurine).

VINCA ALKALOIDS AND OTHER C-MITOTIC ARRESTERS

The vinca alkaloids, colchicine and its derivatives, griseofulvin, and podophyllotoxin have been considered as a class of agents, since there is considerable evidence that in most respects their biochemical mechanisms of action are similar if not identical. Colchicine has been known, in the form of preparations of *Colchicum autumnale,* since antiquity, and is still of great value in the treatment of acute episodes of gout. It has also been used as an antineoplastic agent. A monograph (1) devoted to this agent appeared in 1955, and it has been considered in detail in a review in 1963 by Dustin (2). The vinca alkaloids were discovered as the result of the testing of extracts of *Vinca rosea* Linn. for hypoglycemic activity. This and other aspects of the isolation, chemistry and early clinical evaluation of these compounds, have been described rather completely elsewhere (3, 4). Griseofulvin, an effective antifungal agent for skin infections, isolated from *Penicillium griseofulvin* Dierckx, like colchicine and the vinca alkaloids produces metaphase arrest in mammalian cells (5), although relatively high dose levels are required to cause this effect. Podophyllotoxin is an agent isolated from the resin of *Podophyllum peltatum* Linn., a material long used in popular medicine for the treatment of warts. This compound and some derivatives have received limited clinical trials in neoplastic disease; much of the relevant literature was reviewed in 1953 (6).

The most characteristic biological effect of all of these compounds is arrest of mitosis during metaphase; the duration and extent of mitotic arrest by a number of these agents have been compared by Palmer, Warren & Simpson (7, 8) and by Cardinali *et al.* (9), among others. In general, in this regard vinblastine appeared to be the most active alkaloid. In addition to mitotic arrest, these agents produce other nonmitotic effects, including displacement of the cell nucleus and anisodiametry of the cytoplasm, especially in mast cells (10, 11). Although the metaphase arrest produced by demecolcine (Colcemid), vinblastine, and griseofulvin in *Pectinaria gouldi* oocytes was readily reversible, only partial recovery was attained with vincristine and podophyllotoxin after removal of drug (12). Reversibility of metaphase arrest and reappearance of microtubules around the centrioles, centromeres and chromosomes, have also been described in Earle's L cells treated with vinblastine (13). In the case of human KB cells in culture, Taylor (14) was able to correlate retention of intracellular colchicine with the duration of mitotic arrest. Studies of this type, with tritium-labeled colchicine or vinblastine, have been undertaken recently by several groups using a wide range of biological material. Borisy & Taylor (15, 16) have examined mammalian brain and other tissues, KB cells, flagellated cells and echinoderm oocytes, and have found a correlation between the degree of binding of colchicine and the amount of microtubular elements.

A protein that is apparently the target site for binding of the alkaloid was partically purified from sperm flagella (17). Wilson & Friedkin

(18) have described similar evidence for binding of colchicine with a protein from grasshopper embryos. In addition, in sarcoma 180 ascites cells, Creasey (19, 20) has shown that vinblastine, in addition to colchicine, is bound by a supernatant protein, and that there is an interaction among various antimitotic agents suggestive of the presence of two sites with different binding properties. The essential conclusion of all three investigations is that antimitotic agents bind to a low molecular weight protein that occurs primarily in the supernatant fraction obtained by centrifuging cell homogenates at 100,000 x g. This protein appears to be a constituent of all cell structures containing microtubular elements. Since these elements occur not only in mitotic spindles (21), but also in a wide variety of cells and cellular organelles, including the flagella of plant and animal sperms (22, 23), human polymorphonuclear leukocytes (24), cellular microspikes and cortical cytoplasm (25), and neuronal processes (26), any agent that binds to microtubular protein would be expected to interfere with the dynamics of formation and function of organelles derived from such subunits. Thus, mitotic arrest would be merely one aspect of an effect whose other manifestations might include changed motility or intracellular viscosity, impaired nucleo-cytoplasmic interactions, or cessation of phagocytosis. Such effects could explain the relief of acute gouty episodes induced by treatment with antimitotic agents that may function by reducing the activity of inflammatory cells (27).

Reversible phenomena such as mitotic arrest may not, however, be adequate by themselves to explain cytotoxic action. Indeed, a lack of correlation between mitotic arrest and cytotoxicity was observed in a study of the effects of vinblastine and vincristine in man and in the rat (28). Among the earlier studies of the vinca alkaloids, the finding that certain amino acids, including glutamic acid, partially protected cells against both cytotoxic (29) and mitotic (30) effects suggested that these agents were involved in amino acid metabolism. Although a similar protective effect also has been found in some biochemical studies (31), the binding of antimitotic drugs (20, 32) and their uptake by cells (33) is not affected by glutamic acid. It has not been possible to relate this interaction with amino acids to other aspects of the action of antimitotics.

Another finding, suggestive of interference with purine nucleotide biosynthesis, is the reduction in urinary excretion of 4(5)-amino-5(4)-imidazolecarboxamide that occurs after administration of vinblastine (34).

Both vincristine and vinblastine modify respiration and lactic acid production by cells *in vitro* (35, 36), but the significance of this in terms of the biochemical mechanism of action of these agents is not clear in view of the production of the same type of effect by a variety of unrelated chemotherapeutic agents (37).

The most productive of the biochemical approaches to the antimitotic action of these compounds has been a study of their effects on the synthesis of nucleic acids. Colchicine and demecolcine (Colcemid) inhibit the forma-

tion of DNA *in vitro* by the skin of the ears of guinea pigs (38). Similar inhibitory action of colchicine on DNA synthesis in several normal mammalian tissues *in vivo* has also been reported (39). In *Tetrahymena pyriformis,* vincristine causes a marked reduction in incorporation of thymidine into DNA, with little effect on the formation of RNA or protein; riboflavin and flavin mononucleotide were able partially to protect against this inhibitory action (40). In rat thymus cells *in vitro,* vinblastine is an active inhibitor of DNA biosynthesis; its activity in this system is closely approached by vinleurosine, but exceeds that of vincristine and vinrosidine (41). The inhibitory action of vinblastine was more marked on the synthesis of the purine bases of DNA than on the pyrimidines (42), a finding that could relate to the changes in 4(5)-amino-5(4)-imidazolecarboxamide excretion mentioned previously (34). In other studies, nucleolar DNA has been suggested as the fraction most sensitive to the inhibitory effect of vinblastine (43). Among normal tissues such as rat spleen and bone marrow (44) and regenerating liver (45), vinblastine primarily inhibited DNA synthesis, with small effects on the formation of nuclear RNA. On the other hand, in Ehrlich ascites cells, vinblastine only inhibited the synthesis of DNA at high concentrations *in vitro* (46), but not in these cells isolated from tumor-bearing mice treated with the drug. In contrast, however, both colchicine and vincristine were active inhibitors of the biosynthesis of DNA *in vivo* (47).

In this same tumor line, Creasey & Markiw (46) found that soluble RNA was the macromolecular species whose synthesis was most sensitive to vinblastine when this agent was administered to the tumor-bearing animals. Love (48) has also characterized disturbances in ribonucleoproteins as being a prominent feature in Ehrlich cells treated with colchicine. Decrease in RNA content, without change in the levels of DNA, has been observed in both rat thymus and lymphosarcoma P1798 as a result of administration of vinblastine (49). Interference with incorporation of precursors into the RNA of human leukemic cells has also been noted (47, 50). These effects on nucleic acid synthesis are apparently not the result of some indirect action, such as release of adrenal steroids (51), but more probably arise from direct inhibition of polymerase enzyme systems, as in the case of RNA nucleotydyltransferase (52, 53). In this connection, reports of binding of colchicine by DNA (39), and of griseofulvin by the RNA of sensitive fungi (54), are suggestive of the mechanisms involved in such inhibition.

Interference with the metabolism of the nucleic acids would be expected to disturb protein synthesis, and such effects have been reported both *in vivo* (31) and *in vitro* (50, 55). In addition, the uptake of glutamic acid by tumor cells *in vitro* is inhibited by vinblastine (55).

Another transport function that is apparently subject to interference by the vinca alkaloids is the entry of uridine into tumor cells; vinblastine inhibits this process in both the Ehrlich carcinoma and human leukosarcoma

cells *in vitro* (47). The uptake of deoxyribonucleosides under similar conditions, however, is not affected by this agent.

In some recent unpublished studies, Creasey has found that vinleurosine and vincristine markedly inhibit the incorporation of acetate into the phospholipid fractions of sarcoma 180 cells, with little or no effect on neutral lipids. Such an effect might be relevant to the neurological toxicity, so characteristic of vincristine (3), which is associated with lesions in the phospholipid-rich myelin sheath (56). On the other hand, vincristine also reduces the incorporation of uridine into all fractions of RNA in mouse brain (57), so that the biochemical basis for neurological toxicity is still uncertain.

The importance of other findings to the biological effects of these agents remains to be determined. For example, Bariety & Gadjos (58) have reported that in the rabbit administration of vinblastine led to a significant elevation of the blood levels of iron and copper, and Moncrief & Heller (59) have suggested that the vinca alkaloids, which possess $-COOCH_3$ groups at positions 3 and 18′, may function as acylating agents.

The synthesis of tritiated colchicine has been described in reports from two laboratories (14, 60). Tritiated vinblastine has been prepared both by the Wilzbach technique (61, 62) and by the use of tritiated acetic anhydride (63). Studies of the distribution of vinblastine in rats have shown that maximum blood levels are attained in 1 to 2 hr with 75 per cent of the activity in the buffy coat fraction. Only about 6 per cent of the dose of this alkaloid was excreted in the urine in a 24-hr period, the major route for excretion apparently being through the bile duct (61–63).

The antineoplastic activity of a number of colchicine derivatives, including demecolcine (Colcemid), has been discussed in the review by Dustin (2); furthermore, a comparison of some basic colchicinamides has been made recently (64). The synthesis and activity of 4-acyl analogues of vinblastine, notably vinglycinate, have been described (65). Biological activity, approximately 10 per cent of that of vinblastine, has been ascribed to dihydrovinblastine (66).

N-Isopropyl-α-(2-Methylhydrazino)-p-Toluamide

Certain derivatives of methyl hydrazine have shown considerable antineoplastic activity in transplantable neoplasms of animals and in malignancies of man; the compound that has found the greatest clinical utility is procarbazine (N-isopropyl-α-(2-methylhydrazino)-*p*-toluamide; MIH; Natulan) (67, 68). The metabolism of this agent has been extensively investigated; in man and the dog, rapid equilibration of MIH between the plasma and the cerebral spinal fluid occurs following parenteral administration (69). About 25 per cent of the dose of MIH given to man and rodents is excreted in the urine within 24 hr. Less than 5 per cent of the dose of this agent is excreted unchanged; the majority in the urine is present as a me-

tabolite, N-isopropylterephthalamic acid (69, 70). The appearance of this urinary metabolite is in agreement with a degradative scheme based upon experiments by Berneis *et al.* (71). Initially, MIH is oxidized to yield an azo derivative that has been identified in blood (70) with the coproduction of hydrogen peroxide. The envisioned transformations include conversion of the azo derivative to a hydrazone which is subsequently cleaved to an aldehyde, *p*-isopropylcarbamylbenzaldehyde, and then oxidized further to N-isopropylterephthalamic acid. Methylhydrazine has been proposed as a product of these reactions; however, its existence *in vivo* has not been proved. Demethylation of MIH has been reported by several laboratories (72–78); the precise metabolite that donates the one carbon fragment remains to be determined. The formation of CO_2 from MIH labelled in the methyl group has been observed (73–75); this process takes place in the microsomal fraction of the liver (74). Dost & Reed (76) have reported the conversion of both MIH and methylhydrazine to methane and CO_2 in rats. Weitzel, Schneider & Fretzdorff (77) have shown that cytostatic methylhydrazine derivatives split off formaldehyde after mild dehydrogenation *in vitro;* furthermore, identification of formaldehyde in rodents treated with MIH has been reported (75). Methylamine also has been described as a product of the metabolic transformation of both MIH and methylhydrazine (75), in this regard, Baggiolini & Bickel (78) found that both MIH and methylhydrazine inhibit the conversion of methylamine to CO_2 *in vivo.*

The biochemical basis for the growth-inhibitory activity of MIH is unknown. Rutishauser & Bollag (79) showed that 1-methyl-2-benzylhydrazine phosphate markedly prolongs the duration of interphase in the Ehrlich carcinoma; this phenomenon is accompanied by chromatid breaks, which occur apparently either during or after the synthesis of DNA. The involvement of nucleic acid metabolism in the mechanism of action of MIH gains further support from the observation that several carcinostatic derivatives of methylhydrazine possess the ability to degrade DNA *in vitro* (71, 80). The mechanism of this degradation appears to involve the oxidation of MIH to hydrogen peroxide which is responsible for splitting the DNA molecule into small fragments without substantial destruction of the double helical structure. Inhibition of the synthesis of DNA, as well as blockade of the formation of RNA and protein, occurs in neoplastic cells treated with MIH (81–85). Essentially equal inhibition of the syntheses of DNA and RNA was induced by MIH (81, 83); MIH solutions required a period of aging to exert inhibitory effects *in vitro* (83), while freshly prepared solutions caused blockade of nucleic acid synthesis *in vivo* (81). The inhibitory component that resulted from the aging process did not appear to be hydrogen peroxide. The inhibitory effect of MIH upon the synthesis of DNA could be reversed by washing the cells free of drug (83); this result correlates with the finding that no extensive breakdown of DNA occurred in tumor cells treated with MIH (81, 83). Blockade of DNA synthesis could not be accounted for by inhibition by MIH of the enzymes thymidine kinase, thymidine

monophosphate kinase, or DNA polymerase (81); however, relatively high concentrations of hydrogen peroxide and formaldehyde, both products of the catabolism of MIH, inhibit both DNA and RNA polymerase (84). In addition, formaldehyde retards the synthesis of nucleoside triphosphates, a lesion potentially capable of contributing to a sequential blockade of nucleic acid synthesis. N-Hydroxymethyl derivatives and formylhydrazine, compounds postulated to be potential metabolites of MIH (77), also inhibited DNA and RNA polymerase (84).

The action of this agent on the biosynthesis of protein appears to be delayed until after the onset of inhibition of the formation of nucleic acids (81, 83), suggesting that blockade of protein synthesis is a secondary event. The mechanism of this inhibition has been investigated in liver derived from MIH-treated rodents (85). In this system MIH did not appear to affect the following parameters: (*a*) the number of polysomes; (*b*) the capacity of the ribosomes to accept polyuridylic acid; (*c*) the ability of tRNA to accept amino acids; (*d*) the loading of the tRNA by the pH-5 enzymes; and (*e*) the binding of amino acyl-tRNA to polysomes. The incorporation of amino acids was depressed, however, when polysomes from MIH-pretreated animals were employed, indicating that the polysome fraction is the damaged component; this effect was accompanied by a relative insensitivity of the polysome fraction to breakdown, suggesting a drug-induced change in conformation. The finding that MIH binds to the polysome fraction supports this contention (85); the results of these studies suggest that the intact MIH molecule was bound.

The action of MIH on cellular respiration and glycolysis has been investigated (83, 86); little or no inhibition of these processes was induced by this agent in neoplastic cells. Maximum cell death appears to occur in neoplastic populations after the cells recover from the inhibitory action of the drug on the syntheses of nucleic acids and protein; this period corresponds with the development of a metabolic imbalance characterized by an accumulation of RNA and protein and an increase in cell volume (81).

The role of methylation of the nucleic acids by MIH in the carcinostatic potency of this agent remains to be determined (72, 87, 88). Incorporation of radioactivity from the methyl group of MIH into adenine, guanine, and thymine of the nucleic acids indicates that at least a part of the one carbon unit derived from N-demethylation of MIH enters the normal pathways of intermediary metabolism. In addition, 7-methylguanine has been isolated from both DNA and RNA of neoplastic ascites cells (87) and thymine, 5-methylcytosine, 1-methylguanine, 7-methylguanine, and possibly 1-methyladenine have been isolated from cytoplasmic RNA (88). Furthermore, MIH caused inhibition of transmethylation of cytoplasmic RNA from methionine; this correlated with preliminary experiments suggesting that pre-treatment of tumor-bearing animals with methionine prior to administration of MIH prevents the antineoplastic properties of this compound (88).

Cytosine Arabinoside

Cytarabine (cytosine arabinoside; ara-C; 1-β-D-arabinofuranosylcytosine; arabinosylcytosine) is a synthetic member of a class of nucleosides having D-arabinose as the pentose moiety. The related derivatives, arabinosylthymine and arabinosyluracil, occur naturally in the sponge *Cryptotethia crypta* (89). Certain aspects of the arabinosides, concerned with chemotherapy and a possible role of these agents in aging processes, have been considered by Cohen (90).

Arabinosides have been prepared by several synthetic routes. Fox, Yung & Bendich (91) described a process involving tritylation, formation of O-mesyl derivatives, and hydrolysis with methanolic NH_3 to give cyclic compounds which undergo acid hydrolysis to arabinosides. The phosphorylation of ribonucleosides with polyphosphoric acid has proved to be a convenient method for the synthesis of arabinoside diphosphates; the success of this procedure derives from the cyclisation that occurs during the reaction (92, 93). Another synthetic pathway to arabinoside derivatives involves a transformation of xylofuranosides (94). The preparation of arabinoside phosphates, by reacting suitably protected nucleosides with cyanoethyl phosphate, has been described by Smrt (95). Wempen *et al.* (96) have prepared a number of N_4-substituted derivatives of ara-C by a thiation procedure followed by alkylation and then reaction with nucleophiles.

Ara-C inhibits the growth of a remarkably wide range of rodent tumors, giving a high incidence of "cures" (97); such data have led to extensive clinical trials, especially in the treatment of leukemia. The compound also inhibits the growth of other proliferating tissues, such as regenerating bone marrow (98) and embryos (99), sometimes with production of developmental abnormalities in the latter, as well as the replication of DNA-containing viruses (100). Cytological changes produced by ara-C include chromosome aberrations and deletions (101–103), nuclear bleb formation (104), and changes in shape, granularity, and nucleolar size (105).

The metabolic fate of ara-C has been the subject of intensive investigation because of its relevance to the duration and degree of effectiveness of the drug. In aqueous buffered solution, ara-C undergoes significant deamination to arabinosyluracil (ara-U); this process is catalyzed by anions such as bisulfite, phosphate, and lactate (106). *In vivo,* however, such spontaneous deamination does not appear to be significant, enzymatic deamination being dominant. The ability to deaminate ara-C is widely distributed, being found in bacteria (107) and in many mammals (108–110). In man, deamination is particularly extensive, and blood levels of ara-C are not detectable for more than 5 to 20 min after injection (111). The enzyme responsible for catalyzing this reaction is a pyrimidine nucleoside deaminase that has been best characterized in mouse kidney (112) and human liver (113). The enzyme acts on a relatively wide range of nucleoside substrates, although it does not appear to deaminate most aminopyrimidine bases or nucleotides.

The ability of various derivatives of ara-C to function as substrates for the deaminase, as well as to act as inhibitors of the reaction, has been compared by Dollinger *et al.* (114) and by Camiener (113). Despite the rapid deamination of this compound, and the ease with which the arabinoside linkage is cleaved by bacteria (115), in man (111) and in other mammals (109), further metabolism of ara-U does not occur.

Since the early work of Chu & Fischer (116), it has been known that metabolic phosphorylation is a necessary prerequisite for ara-C to function as an inhibitor of cell growth. There is indeed a close correlation between the ability of both human and mouse leukemic cells to accumulate the nucleotide forms of ara-C, and their response to the drug (117). In sensitive cells, both murine (118–120) and human (111), the major proportion of intracellular drug is in the form of nucleotides. Resistance to this agent is accompanied by a profound fall in the ability of the cells to phosphorylate both ara-C and deoxycytidine. That the same kinase is responsible for the phosphorylation of both nucleosides is evident from studies in mouse tumor cells (118–120) and human leukemic leukocytes (121); in these investigations phosphorylation of ara-C was almost totally inhibited by deoxycytidine. This phenomenon almost certainly forms the major basis for the antagonism of the antiviral and antitumor effects of ara-C by deoxycytidine, seen both *in vitro* (116, 118, 122) and *in vivo* (123). In contrast, uridine potentiates the antineoplastic action of ara-C, apparently because the kinase enzyme phosphorylates ara-C at twice the rate with uridine triphosphate in place of adenosine triphosphate as the phosphate donor (124).

The possible sites of the metabolic lesions induced by ara-C have been the subject of considerable study and speculation. Early data, notably that of Chu & Fischer (116, 118), pointed to an inhibition of the ribonucleotide reductase reaction that leads to the formation of deoxycytidine diphosphate by phosphorylated derivatives of ara-C. Thus, it was envisioned that administration of deoxycytidine relieved the effects of ara-C both by competition for the kinase enzyme and by conversion to deoxycytidine nucleotides required for the formation of DNA. Evidence from two areas, however, has shown this view to be unduly simplified. First, the antagonism by deoxycytidine is not a simple competitive relationship, since irreversible damage by ara-C occurs (125), and second, ara-CTP is only a weak inhibitor of the ribonucleotide reductase from the Novikoff ascites tumor, exhibiting potency no greater than deoxycytidine triphosphate (126). However, since inhibition by ara-C of the uptake of such precursors as cytidine and thymidine, but not deoxycytidine, into DNA has been a consistent finding in tissue culture (116, 127–130) and in human leukemic cells (121, 131), the levels of ara-C phosphates achieved in whole cells may be high enough to exert a moderate degree of pseudo-feedback inhibition of the reductase enzyme. That other factors are also involved in the inhibitory effect on DNA synthesis is shown by reports of the inhibition by ara-CTP of DNA polymerase from Ehrlich ascites cells (132) and from calf thymus and bo-

vine lymphosarcoma (133). In these preparations, the inhibition was kinetically competitive with deoxycytidine triphosphate.

The production of irreversible lethal changes in cells, however, would appear to demand something more than competitive inhibition of enzymic activities. Accordingly, considerable attention has been directed to the possible incorporation of ara-C into the nucleic acids. There is evidence for such incorporation in mammalian cells in tissue culture (118, 125, 127) and in human leukemic cells (111, 121); in the most critical of these studies isolation of ara-C has been obtained following hydrolysis and enzymatic degradation of nucleic acids. In L5178Y cells the incidence of irreversible inhibition of cellular reproduction was correlated with incorporation of ara-C into RNA (125). The amount of labelled ara-C present in both RNA and DNA is extremely small, indicating, when one considers the relatively high levels of ara-CTP in the acid-soluble fraction, that the nucleotide, even under the most favorable circumstances, is a very poor substrate for the polymerase enzymes. This would appear to be the reason for the repeated negative findings with regard to incorporation of ara-C into the nucleic acids in studies with RNA and DNA polymerase preparations (133, 134).

The biochemical changes described as occurring in cells treated with ara-C are all of the type associated with unbalanced growth (121, 127, 128), namely reduced DNA synthesis in the presence of continuing formation of RNA and protein. In accord with this concept, increased levels of deoxycytidine kinase have also been described in kidney cells exposed to ara-C (130).

Recent interest in ara-C as a chemotherapeutic agent has centered on the possibilities offered by the relative specificity of this agent for the S-phase of the mammalian cell growth cycle (135). This specificity stems from the fact that the major possible sites of action all appear to involve DNA and its biosynthesis. Such an approach, which depends upon the presence of a greater percentage of the population of neoplastic cells in the S-phase as compared to the cells of the most sensitive tissues of the host, as well as the use of ara-C in combination with other agents that conceivably might synergize with it, offers hope for increasing therapeutic efficacy.

6-Methylthiopurine Ribonucleoside

Although 6-methylthiopurine ribonucleoside (6-MMPR), synthesized in 1957 (136), was shown to have less antitumor activity than 6-mercaptopurine (6-MP) (137), there has been renewed interest in the compound because of its lack of cross-resistance in most systems with 6-MP (138). The basis for the absence of cross-resistance to 6-MMPR in 6-MP resistant cells that have a deleted or markedly decreased activity of hypoxanthine-guanine phosphoribosyltransferase, the enzyme which anabolizes 6-MP to a nucleotide derivative, is that 6-MMPR is converted to a 5′-monophosphate by adenosine kinase (138, 139). The properties of mouse and human adenosine kinase in relation to the anabolism of 6-MMPR have

been described recently (140). Phosphorylation appears to proceed only to the 5′-monophosphate level and, therefore, no significant incorporation of 6-MMPR into polynucleotides occurs (138–140). Unlike most purine ribonucleosides, 6-MMPR is resistant to phosphorolytic cleavage (141); this resistance leads to a pattern of unusual intracellular persistence. Thus, for example, the compound, as the 5′-monophosphate, is concentrated in human red cells where it may persist for up to 6 weeks; in these patients, urinary excretion, essentially of unchanged drug and its phosphate ester, proceeded at a daily rate of about 10 per cent of the dose (142). The nucleotide of 6-MMPR exerts a pseudo-feedback inhibition of purine nucleotide biosynthesis *de novo* by inhibiting the activity of phosphoribosylpyrophosphate amidotransferase, as reflected in the formation of formylglycinamide ribonucleotide (138). Of 16 purine nucleotides tested against this enzyme, partially purified from adenocarcinoma 755, the monophosphate of 6-MMPR was the most potent inhibitor (143). Resistance to 6-MMPR in a strain of H.Ep.2 cells in culture was ascribable to loss of adenosine kinase activity (144). Other mechanisms of resistance exist, however, since in a strain of Ehrlich ascites carcinoma resistant to 6-MMPR, the rate of formation of the nucleotide was unchanged; the evidence in this system pointed either to some alteration in the mechanism of interaction of the inhibitor with the phosphoribosylpyrophosphate amidotransferase enzyme, or to a somewhat increased rate of breakdown of the nucleotide (145). There are data suggesting that some of the metabolic alterations induced by 6-MP may be due to the formation of 6-MMPR phosphate. Thus, in H.Ep.2 cells treated with 6-MP, as much as 33 per cent of the derived intracellular nucleotide forms was present as 6-MMPR phosphate (146). This nucleotide was formed by methylation of 6-MP ribonucleotide rather than by methylation of either the free base or nucleoside and subsequent phosphorylation; no demethylation of 6-MMPR nucleotide appeared to occur. The levels of 6-MMPR nucleotide generated from 6-MP in neoplastic cells may well be adequate to account for the inhibition of phosphoribosylpyrophosphate amidotransferase activity by 6-MP (147). Synergistic antitumor effects of 6-MMPR in combination with 6-MP have been described in both L5178Y lymphoma cells in culture (148), and in L1210 (149) and Ehrlich ascites carcinoma (150) *in vivo*. The potentiated response to this drug combination appears to result from a five- to seven-fold increase in the levels of 6-MP nucleotide produced in cells treated previously with 6-MMPR; the increase in the intracellular concentration of 6-MP nucleotide is related to a 6-MMPR-induced increase in the levels of phosphoribosylpyrophosphate and a decrease in the amount of adenine nucleotides (151).

Hydroxyurea

A variety of transplantable neoplasms have been shown to be sensitive to the hydroxylamine derivative hydroxyurea (67). In man, it is moderately active in chronic myelocytic leukemia and may be of transient benefit in

metastatic melanoma. Structure-activity relationships have shown that the -N-OH group is required for inhibitory activity; consequently, the proton in the hydroxyl group may not be substituted (152). Inhibition of the growth of both mammalian and microbial cells by this agent appears to be predominantly due to its capacity to interfere with the formation of DNA; thus, concentrations of hydroxyurea which cause a marked decrease in the rate of biosynthesis of DNA produce little or no inhibition of the incorporation of precursors into RNA or protein (152–164). Corroborating these findings is the observation that hydroxyurea produces a differential lethal effect on cultured Chinese hamster cells that are at different stages in their growth cycle. Thus, cells actively synthesizing DNA at the time of exposure to this agent are lethally damaged, whereas those cells in the G1 phase of growth survive, but are prevented from beginning DNA synthesis. Populations in the G2 period also survive and appear to progress to a stage just prior to the beginning of the next period of DNA synthesis (165). Investigations designed to delineate the site of the metabolic lesion on the DNA biosynthetic pathways have indicated that DNA polymerase, thymidine kinase, and thymidylate kinase are unaffected by hydroxyurea *in vitro* (157); furthermore, the drug does not degrade or cross-link DNA (166). The reduction of ribonucleotides to their corresponding deoxyribonucleotide forms, however, is markedly inhibited (154, 167–170). In accordance with such metabolic blockade, a concentration-dependent decrease in the acid-soluble deoxyribonucleotide triphosphate pool of hydroxyurea-treated *E. coli* cells occurs without significant change in the ribonucleoside triphosphate pool (171). In several systems, the addition of deoxynucleosides either singly or in admixture reversed in part the inhibition of growth (153, 172) and the blockade of the incorporation of thymidine into DNA (152) afforded by hydroxyurea. Studies on the mechanism of inhibition of ribonucleotide reductase have shown that hydroxyurea is not competitive either with the various ribonucleotide substrates (i.e., UDP, CDP, GDP) or the allosteric activator ATP, whereas the inhibition by this agent is partially competitive with the hydrogen donor dithioerythritol and ferrous ion (168–170). The latter effect supports the requirement for metal-binding potential by agents of this type for the capacity to inhibit the growth of mammalian cells (152).

It has been suggested (173–175) that hydroxyurea may act *via* conversion to hydroxylamine; however, direct comparison of these two agents has shown that while hydroxyurea inhibits mainly the biosynthesis of DNA, hydroxylamine interferes with all macromolecular syntheses (i.e., DNA, RNA, and protein). Hence, such a possibility would appear unlikely. Acetohydroxamic acid has been found in the blood of patients with chronic myelogenous leukemia on hydroxyurea therapy (173). In contrast, rodents appear to metabolize about 10 per cent of a dose of hydroxyurea to CO_2 and 50 per cent to urea; the remaining drug is excreted unchanged in the feces (176). Furthermore, drug-sensitive neoplastic lymphoma cells (L1210) do not form any unique metabolites of this agent *in vitro*, supporting the concept that the pharmacological activity is due to unaltered drug.

Since a mixture of deoxyribonucleosides does not reverse the blockade of DNA synthesis afforded by hydroxyurea in some systems (*166*, *177*, 178), an additional unknown site of action other than ribonucleotide reductase has been proposed; however, the lack of reversal by exogenous deoxyribonucleosides may well be due to extensive catabolism of these compounds upon entrance into cells. Evidence has been provided that hydroxyurea inhibits the formation of histones (159), the biosynthesis *de novo* of pyrimidine nucleotides prior to the formation of orotidylic acid (179), and requires for expression of its lethal action in *E. coli* the biosynthesis of protein. Hydroxyurea induces chromosome breaks in several systems (174, 181, 182), as well as direct fragmentation of isolated DNA *in vitro* (183). The mechanisms involved are unknown, but would not appear to be mediated through the generation of hydroxylamine (181).

THIOSEMICARBAZONES

α-Ketoaldehyde *bis*(thiosemicarbazones) possess the capacity to retard the growth of transplanted rodent neoplastic cells. The derivative that has received the most study is 2-keto-3-ethoxybutyraldehyde *bis*(thiosemicarbazone) [KTS; kethoxal *bis*(thiosemicarbazone)]. The antineoplastic potency of KTS is enhanced by the presence of either cupric or zinc ions (184–187); of several different metal chelates of KTS, the preformed cupric chelate [Cu(II)KTS] has been found to be the most potent (184, 188, 189). KTS chelates metals of the first, second, and third transition series; in this regard, with respect to copper and zinc, one molecule of KTS is coordinated to one metal ion to form quadridentate chelates (188, 190–192).

The metabolic process that is most sensitive to Cu(II)KTS is the biosynthesis of DNA; the rate of formation of RNA and protein is also depressed by this agent, but inhibition of these latter functions is less pronounced (*186*, *193–196*). Although both Cu(II)KTS and KTS, itself, inhibit the fabrication of DNA, distinct differences can be detected in the metabolic lesions induced on the biosynthetic pathways to DNA. Thus, the effects of Cu(II)KTS on sarcoma 180 cells are characterized by a pronounced decrease in the rate of incorporation of thymidine into DNA and lesser inhibitions of the utilization of formate, deoxycytidine, and uridine for the formation of the pyrimidine nucleotides of DNA. The enzymatic site most sensitive to the inhibitory activity of Cu(II)KTS in sarcoma 180 appears to be the enzyme thymidine kinase (195), whereas in KB cells thymidine kinase and deoxycytidine kinase are equally sensitive to the cupric chelate (196). In contrast, the ligand KTS does not decrease the rate of incorporation of thymidine into DNA but causes marked inhibition of the utilization of formate, deoxycytidine, and uridine for the formation of DNA (195). These effects can be accounted for by KTS inhibition of the enzymes thymidylate synthetase, 5,10-methylenetetrahydrofolate dehydrogenase, and dihydrofolate reductase (197). Sufficient intracellular concentra-

tions of KTS are achieved in neoplastic cells to inhibit these enzymes (197).

The cellular toxicity of Cu(II)KTS is essentially caused by copper. The relatively lipid-soluble Cu(II)KTS complex effectively serves to transport and deposit copper at sensitive sites within cells. Dissociation of Cu(II)KTS occurs within neoplastic cells treated with the cupric chelate, presumably because of the greater avidity of copper for some of the cellular constituents; this phenomenon is followed by the cellular elimination of the ligand portion of the molecule and the much slower expulsion of copper (195). The findings obtained, using a variety of isotopic precursors of DNA as biochemical probes, suggested that the intracellular localization of copper derived from Cu(II)KTS differed from that derived from $CuCl_2$ (195).

Similar tests, although limited, have been conducted with the zinc chelate [Zn(II)KTS]. Although it was reported that dietary supplements of zinc acetate lowered host-toxicity without increasing antineoplastic potency of KTS (187), the direct administration of Zn(II)KTS to tumor-bearing animals produced carcinostasis (189) as well as pronounced inhibition of the synthesis of DNA, RNA, and protein (198).

A role for thiol-containing compounds in the mechanism by which KTS causes growth inhibition has been shown in *Saccharomyces carlsbergensis*. In this system, toxicity of KTS is prevented competitively by glutathione, whereas the inhibition of this organism produced by Cu(II)KTS is not antagonized by the thiol (199). A relationship between certain water-soluble vitamins and *bis*(thiosemicarbazones) also appears to exist; thus, decreased levels of thiamine and pantothenic acid were produced in livers of rats treated with high concentrations of KTS (200). Supplementation of the diet of tumor-bearing rats with excess thiamine and pantothenic acid reduced the restriction of the growth of the host induced by KTS and enhanced the tumor-inhibitory potency of this agent. The carcinostatic activity of KTS, and of the related derivative methylglyoxal *bis*(N^4-methylthiosemicarbazone), was enhanced in pyridoxine-deficient animals; no direct relationship was envisioned, since the inhibition of the growth of *S. carlsbergensis* by these agents was not prevented by pyridoxal, pyridoxine, or pyridoxamine, and methylglyoxal *bis*(N^4-methylthiosemicarbazone) did not decrease the level of vitamin B_6 in neoplastic cells (201, 202). The finding that the related agent pyruvaldehyde *bis*(thiosemicarbazone) was more active against tumors propagated in male animals than in females suggested a relationship between the sex hormones and the activity of this agent (203).

A wide spectrum of α-(N)-heterocyclic carboxaldehyde thiosemicarbazones has demonstrated pronounced antineoplastic activity when tested against a variety of transplanted tumors; the most active of these derivatives are 1-formylisoquinoline thiosemicarbazone (204, 205), its 5-hydroxy derivative (206), and both 3-hydroxy-2-formylpyridine thiosemicarbazone, and 5-hydroxy-2-formylpyridine thiosemicarbazone (207, 208). The poten-

tial for a conjugate N*-N*-S* tridentate or bidentate ligand system was found to be a common feature of compounds with carcinostatic potency (204); attachment of the carbonyl group in a position α to an unencumbered heteroaromatic nitrogen atom was required. These compounds are excellent coordinating agents for a number of transition metals including divalent iron, cobalt, nickel, copper, zinc, and manganese (207). The exceptionally strong affinity for ferrous ions *in vivo* was demonstrated by the finding that administration of a 100 mg/kg dose of pyrazine-2-carboxaldehyde thiosemicarbazone to mice removed about 11 μg of iron in 24 hr (209). The biochemical basis for the growth-inhibitory activity of 1-formylisoquinoline thiosemicarbazone appears to be due to inhibition of the biosynthesis of DNA; blockade of the formation of RNA and protein by this agent also occurs, but these processes are less sensitive to drug-induced inhibition (210–212). Interference with the fabrication of DNA results from direct inhibition of the enzymatic reduction of ribonucleotides to deoxyribonucleotide forms; the potency of these compounds as inhibitors of the ribonucleotide reductase appears due, in part, to their capacity to bind iron (212, 213). A similar mechanism of action appears to be operative with both 3-hydroxy-2-formylpyridine thiosemicarbazone and 5-hydroxy-2-formylpyridine thiosemicarbazone (214).

Asparaginase

Several experimental neoplasms have a nutritional requirement for L-asparagine (215–221). Such knowledge, coupled with the identification of the tumor inhibitory factor in guinea pig serum as L-asparaginase (222–224), prompted the testing of this enzyme on a variety of neoplasms. Carcinostatic properties against several tumors, particularly certain lymphocytic tumors of both animals and man, have been demonstrated for L-asparaginase from several sources. A good correlation appears to exist between the requirement of neoplastic cells for an external source of asparagine and their susceptibility to treatment with L-asparaginase. Following administration of L-asparaginase to both tumor-bearing animals and patients with malignancy, a profound lowering of the levels of asparagine in the blood and tissues occurs (225–227). Determination of the levels of L-asparaginase in brain and blood at relatively short times after administration of the enzyme to experimental animals with intracerebrally injected lymphoma cells showed no appreciable levels of this enzyme in the brain of treated mice, despite relatively high blood levels of L-asparaginase in the treated animals (228). The effectiveness of L-asparaginase against this tumor located in the central nervous system would, therefore, appear to be due to a deprivation of asparagine in brain by equilibration with the low systemic levels of this amino acid.

The requirement for an external source of asparagine by certain neoplastic cell lines appears to be due to a limited capacity of these cells to fabricate sufficient L-asparagine for their requirements for growth and

function. Accordingly, transplanted murine tumors that are unresponsive to L-asparaginase possess high levels of the enzyme asparagine synthetase, whereas, in contrast, asparaginase-sensitive neoplasms have little or no asparagine synthetase activity. Furthermore, resistant variants from L-asparaginase-sensitive populations have a relatively high capacity for the synthesis of asparagine (229–232). The activity of asparagine synthetase is low in normal mouse spleen, lymph node, thymus, liver, and kidney, whereas activity is greater in brain and testes. Although the levels of the synthetase enzyme in many tissues of the mouse are considerably lower than those of resistant neoplasms, the metabolic demands for asparagine are apparently met by synthetic capabilities, since no unique organ toxicity has been reported in animals receiving L-asparaginase. It has been reported that large increases in asparagine synthetase activity were induced in L-asparaginase-resistant neoplasms following exposure to this enzyme; lesser and more transient elevations of synthetase activity were observed in spleen and in an L-asparaginase-sensitive tumor (232). The finding that resistant cells respond to deprivation of exogenous asparagine by increasing the synthesis of this amino acid (230) suggests that derepression of the formation of asparagine synthetase is a result of a deficiency of asparagine.

The precise consequences of an inadequate supply of L-asparagine are unknown; the metabolic transformations which this amino acid undergoes in mammalian tissues are: utilization for synthesis of protein, conversion to aspartate by hydrolysis, and conversion to α-ketosuccinamic acid via transamination (233). Accordingly, the fabrication of protein in a cell line requiring an exogenous source of asparagine was impaired, while protein synthesis in a subline with greater asparagine synthetase activity was not decreased, by a deficiency of an external source of asparagine (230, 234). Furthermore, the utilization of amino acids for the formation of protein is markedly inhibited by L-asparaginase in sensitive neoplasms (225, 235, 236); this phenomenon is coupled with changes in the intracellular levels of other amino acids, particularly the accumulation of aspartic acid (225). L-Asparaginase-resistant populations not only have higher asparagine synthetic capabilities than sensitive cells, but also appear to be able to utilize endogenously produced asparagine more effectively for protein synthesis (225). Regression of tumor growth induced by treatment with L-asparaginase is preceded by an increase in the activity of alkaline ribonuclease of the post-mitochondrial cell fraction; acid ribonuclease activity of this fraction is not increased by enzymic treatment (237). This phenomenon may be in part responsible for the delayed inhibition of RNA synthesis that occurs in susceptible neoplasms treated with asparaginase (235, 238).

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