The Employment of Combinations of Drugs in the Chemotherapy of Neoplasia: A Review

ABRAHAM GOLDIN AND NATHAN MANTEL

(Laboratory of Chemical Pharmacology and Biometry Branch, National Cancer Institute, * Bethesda, Md.)

Studies on the effectiveness of drug combinations have intrigued pharmacologists for decades. Combinations of drugs have been used in attempts to attain enhanced biological effects. The range of pharmacological interests in which drug combinations have been studied is diverse, including analgesics, insecticides, fungicides, antibiotics, plant and animal hormones, cellular poisons, etc. The recent review by Veldstra (76) may be consulted for the detailing of the numerous areas of interest and for references on drug combinations pertaining to phenomena of synergism and potentiation.

The methods currently employed in investigating the effectiveness of drug combinations in the chemotherapy of neoplasia represent an outgrowth and development of earlier methods of combination drug research. The early medical interest in the employment of drugs in combination was in an attempt to improve therapy. The general concept was that, by the employment of two drugs, lower doses of each could be used, undesirable toxic side effects would be reduced, and there would be a greater margin of safety. Alternatively, drugs would be used in combination in order to take advantage of the separate effects of each, e.g., the rapidity of onset of one drug and the prolonged duration of the other. The early trials with drug combinations were somewhat empirical, with relatively little attempt at quantification. The emphasis, however, was clearly on attaining "therapeutic synergism," that is, the achieving of better therapies by employing drug combinations. In the current presentation a subject of primary emphasis will be "therapeutic synergism" and its quantitative aspects.

The increasing use of drug combinations in non-therapeutic situations such as in mixtures of insecticides, fungicides, or other poisons has tended to lead the evaluation of effectiveness of drug combinations in a direction different from that applicable in the therapeutic case. In place of evaluation in terms of improvement of therapy, drug combinations were evaluated on the basis of the extent of biological effect produced. Usually also, attempts were made in such cases to reduce the amount of material required to achieve a desired effect; e.g., a designated percentage kill of an insect population. In such studies there was not the corresponding problem of the therapeutic case, in which one has to consider the effect of the drug combination on both host and parasite. In certain therapeutic situations, however, the approaches used in terms of reduced material requirements were reasonable. In the early days of the use of penicillin, for instance, it was of practical value to devise methods for reducing the requirements for a drug in such short supply.

The methods for studying drug combinations in nontherapeutic situations have been characterized by the development of mathematical models for the concerted behavior of drugs, with accompanying mathematical concepts of synergism, potentiation, and antagonism. A mathematical discussion of the toxic action of mixtures of poisons is given by Finney (16). Various models were devised to describe the combined action of mixtures of poisons including: (a) "similar joint action," whereby the modes of action of the drugs are similar; (b) "independent joint action," whereby the components of
the mixture act independently on the organism; and (c) "synergism" and "antagonism," whereby the potency of the mixture is greater or less than can be accounted for by the potencies of the individual drugs (8, 16). These and other models (15, 16, 55) provided a mathematical basis for investigation of nontherapeutic synergism.

The development of mathematical concepts of synergism, with the primary emphasis frequently on the employment of reduced amounts of material, resulted in still greater shifts away from the original idea of improved therapies. Further, these methods tended to become established as the routine and proper ways for investigating synergism in all cases.

Interest in the effectiveness of therapeutic drugs in combination received new impetus with the advent of antibiotics such as penicillin and the sulfonamides (89). The methods used, however, in evaluating these combinations have been quite diverse and generally of somewhat limited applicability to the therapeutic case.

The range of methods used in investigating antineoplastic drug combinations will be discussed below. These include methods which represent outgrowths of procedures used in toxicological studies and also quantitative methods used by the authors which emphasize the principle of improvement of therapy.

SYNERGISM

In investigations of the joint action of agents, attempts to demonstrate the presence or absence of synergism have been based on various concepts of synergism. In this review investigations of combination chemotherapy of neoplasia will be discussed in terms of the concept of synergism employed, and for this purpose a preliminary review of these concepts is essential.

DEFINITIONS

The diversity of definitions of synergism and potentiation (and antagonism) is traceable to the historical shifts in emphasis indicated above. Various definitions relating to synergism and potentiation have included:

1. Synergism is the "co-operative action of discrete agencies such that the total effect is greater than the sum of the two effects taken independently;—the opposite of 'antagonism.'" (79).

2. Synergism is a quantitative change in the sense of "increasing the efficiency." The term "potentiated summation" is used "where the combined action is greater . . . than could be calculated" (73).

3. Synergism is "positive summation." The term "potentiation" is employed for instances in which the combined action of two drugs is greater than that which can be anticipated from the sum of their individual actions (39).

4. "Synergism implies the ability of two antimicrobial drugs acting together to increase markedly the rate of early bactericidal action, as compared to the rate with either drug alone, and to kill greater numbers of bacteria or to cure experimental or clinical infections more effectively than could be expected from simple algebraic summation of single drug effects" (90).

5. Veldstra (76) used Webster's definition of synergism translated into molecular terms: "The combination effects a certain response with a smaller number of molecules than that required for the most active compound separately, or: in the range of suboptimal concentrations, the effect of a certain number of molecules of this compound is enhanced in the mixture."

6. Rentz (55) defines "sensitization" as synergism obtained with the combination of an active and an inactive compound. He considers "potentiation" as a form of synergism in which both compounds have the same type of activity. Veldstra (76) points out that there is no fundamental difference between Rentz's "sensitization" and "potentiation." On the grounds that "potentiation" means "endowing with power" and since, in synergism, the effectiveness of the power, rather than the power itself (specific activity), is increased, he recommends that the term "potentiation" be dropped.

7. Venditti et al. (77) interpret synergism in a broad sense as meaning the cooperation or effective combination of agents, with the user free to specify the nature of the cooperation in which he is interested. Therapeutic synergism then exists if combination treatment provides an improved therapy. This criterion for synergism was effectively used by Goldin et al. (30). From this point of view, potentiation could be considered a form of synergism in which the cooperation is such as to reduce drug requirements in achieving a specified effect.

MECHANISMS OF SYNERGISM

Although the definitions of synergism vary, basically they refer to two types of investigation. Synergism may be defined and investigated with respect to: (a) mechanisms pertaining to a single response (end-effect) such as the inhibition of a specific enzymatic transformation, destruction of a bacterial population, toxicity, carcinostasis, cytotoxic action, etc.; (b) mechanisms pertaining to the interrelationship of two responses (end-
effects) such as the host-parasite relationship, host-
tumor relationship, narcosis-toxicity relationship, etc. The concept of therapeutic synergism would apply to this category. Both types of study may be conducted at the biochemical or biological level, in accordance with the interests of the investigator.

Current rationales for ways in which synergism may arise, in the main, stem from biochemical considerations. The differences and lack of agreement in definitions of synergism and potentiation become less formidable on consideration of basic biochemical mechanism.

The demonstration by Woods (80) of a competitive relationship between p-aminobenzoic acid and sulfonamide stimulated the study of antagonist-metabolite relationships. Shive (48, 64, 65) developed the methods of inhibition analysis, employing metabolite-antimetabolite relationships, as a biochemical tool in the elucidation of metabolic transformations. Similar methods have been employed in the mammalian organism (19).

Veldstra (76) attempted to translate studies of synergism into molecular terms. Various investigators (14, 40, 62, 68) have stressed the biochemical approach to studies of synergism. Potter (53) showed the importance of the concept of sequential blockade, where two inhibitors act at different loci in a sequence of metabolic transformations. Elion, Singer, and Hitchings (14) emphasized the concept of concurrent blockade, where two inhibitors act in separate systems but block the formation of a common product. Employing multiple biochemical blockade, they took advantage of a knowledge of the sequence of metabolic transformations in the biosynthesis of polynucleotide purine to investigate the predictability of synergism on the basis of mechanism.

In simplest terms, factors which may increase drug effectiveness include: a decrease in metabolite, precursor, or product of enzymatic transformation; a decrease in enzyme; or an increase in level, time, or rate of action of an antagonist.

Antagonist-metabolite relationships with respect to primary loci of action in vital processes may be outlined in simplified form as follows:

Types of multiple biochemical blockade.—1. Sequential blockade: Two drugs act at separate loci in a series of biochemical transformations (53).

\[
I_1 \downarrow \quad I_2 \downarrow \\
A \longrightarrow B \longrightarrow C \longrightarrow D \longrightarrow X
\]

Inhibition of the transformation \( B \rightarrow C \) by inhibitor \( I_1 \) would reduce the concentration of metabolite \( C \) and thereby increase the inhibitory action of inhibitor \( I_2 \) (53). With this type of blockade, the resultant synergism is one in which there is an increase in the inhibitory effectiveness on a molar basis. However, it may be possible to duplicate the effect of the combination of inhibitors by increasing the concentration of the inhibitors individually.

A modified form of this type of inhibition has been reported with aminopterin, which apparently acts at several loci in a metabolic sequence. A priming dose of aminopterin resulted in a marked increase in the toxicity of a second dose administered several hours later (19).

2. Concurrent blockade (14):

a) Two antagonists compete with metabolite in wholly independent metabolic pathways, each of which is essential. The sites of action are different and wholly independent, but the observable biological response, such as retardation of cell growth or cell death, could be the same.

\[
I_1 \downarrow \\
A \longrightarrow B \longrightarrow C \longrightarrow X \\
I_2 \downarrow \\
E \longrightarrow F \longrightarrow G \longrightarrow Y
\]

In this system, since both pathways are essential, blockade of either pathway will result in inhibition. Since interrelationships between the two independent processes may result from the deficiencies of the products, without further biochemical information, no prediction could ordinarily be made about synergism. On the other hand, in a particular situation, multiple biochemical blockade of wholly independent pathways could serve as a basis for demonstrating synergism. It could, for example, exert enhanced effect in a bacterial or tumor cell population in which resistant mutants to an antagonist were present or were selected on treatment. If the resistance occurred as a result of failure of a single antagonist to compete successfully, or because of a decrease in the essentiality of the metabolic pathway, attack by a second antagonist at a wholly independent site could lead to synergism.

b) Two drugs act in parallel pathways which ultimately merge into a common pathway with the formation of a common product.

\[
I_1 \downarrow \\
A \longrightarrow B \longrightarrow C \longrightarrow X \\
I_1 \downarrow \\
D \longrightarrow E \longrightarrow F \longrightarrow X
\]

1) Both pathways are necessary for the formation of the common product. A block of either pathway will reduce the formation of \( X \), and synergistic activity would not, in general, be
expected. Any extent of inhibition with the combination of inhibitors could be duplicated by increasing the concentration of one of the inhibitors.

2) The pathways are alternate, and either one is sufficient for the formation of the common product. Neither inhibitor alone would prevent the formation of product. Employment of the combination of inhibitors would block product formation. This would be a form of synergism in which it would not be possible to duplicate the action of the combination of inhibitors by the employment of the individual drugs.

Other mechanisms.—

1. One of the drugs, itself ineffective, slows down the rate of disappearance of the effective agent. This would tend to maintain a high concentration of the inhibiting agent at the active site, thus allowing a greater total effect to be achieved. This may occur in various ways. Veldstra (76) describes such phenomena as occurring through competition of an active and a less active or inactive analog for "sites of loss." If the less active compound has a higher affinity for the site of loss than the active compound, this will tend to maintain the effective concentration of the active compound. Such sites of loss would include nonspecific adsorption (tissue storage), metabolic detoxication, or excretion. Various examples are cited (76). A similar effect could be achieved mechanically, by creating a drug depot, as when penicillin is administered in beeswax.

2. One of the agents plays a preparatory role, permitting more effective action of the active agent. The inactive agent may increase the activity of the primary agent by overcoming transport barriers. It may increase the solubility of the primary agent, increase permeability, etc. (76). With this type of synergism, it is not usually necessary for the synergist to resemble the antagonist structurally (76).

By the same token, the second drug could interfere with the transport of essential substances requisite for formation of coenzyme or apoenzyme, or essential precursors, metabolites, or products, thereby enhancing the activity of the primary antagonist.

3. Combination treatment could minimize the chance of failure, where resistant cells or organisms occur either spontaneously or by mutation or adaptive responses resulting from application of an inhibitor (11, 13, 46, 50, 76). The likelihood that simultaneous resistance to two agents will occur or arise is minimal. A suggested basis for synergism, where failure of successful antagonism at a primary locus by a single inhibitor occurred, was cited above. Resistance could result in numerous ways, such as through altered permeability, increase in enzyme concentration, alterations in rates of biochemical reactions, and associated changes in rates of cell growth or division, etc. Dependence on an antagonist may also occur (44, 46).

The effectiveness of action of the combination of drugs is contingent on the mode of action of the drugs relative to the mechanism of resistance encountered.

4. Augmented responses may be obtained by alterations of temporal relationships of application of drugs, including alterations of schedules of treatment. Such enhancement of activity does not necessarily require the use of two drugs, but may be observed with a single agent. For example, the total dose toxicity of aminopterin is altered by the interval between treatments, the number of treatments, and the total interval of treatment (19, 20, 24). This mimicking of synergism may have a biochemical basis similar to that which results in synergism in the employment of two agents (19, 51, 52). In testing the synergistic effect of agents given at different times it is necessary to consider the possibility that the improvement is related to the altered frequency of treatment for the combination (77).

5. When, in a therapeutic situation, the toxicity of an effective agent is limiting, a second drug may reduce the toxic effect for the host and achieve this with little or no loss in damaging effect for the tumor or parasite. The resultant effect would be an improved therapy (23). This is discussed in more detail below.

For the synergistic mechanisms suggested above there could be corresponding mechanisms for antagonism (82).

Various modifications of these schemes would be necessary in accordance with the complex biochemical interrelationships which obtain in vivo, as, for example, in the ramifications and interconversions in the synthesis of purine polynucleotide (5, 7). Also, inhibitors may exert their action in different ways with reference to the same enzymatic locus or at separate loci. In addition to competition with substrate for the active site, they may displace coenzyme on the apoenzyme, complex with substrate, or react with the enzyme-substrate complex (41, 42, 86, 84). Incorporation of inhibitors into nucleic acids has been demonstrated (6, 47). Also, the inhibitors may show various degrees of reversibility (1).

The agents may create an unbalance of amino acids (35) or other metabolites (9), thereby resulting in unbalanced synthesis and greater sensitivity to drug action. They may augment inhibition by
overaccumulation of intermediates in synthesis (10) or by interference with "feed-back" mechanisms (34, 83), or they may have other even more subtle actions. Nevertheless, as stressed by Hitchings (14, 36, 37), Potter (53, 73), Skipper (69), and others (19, 62), elucidation of in vivo biochemical pathways could provide a basis for determining loci of attack for the demonstration of synergistic effects.

**Investigations of Synergism**

When the expectation for synergism is based constructively on the known effects of the separate agents, as well as when the combination is considered only on the chance that it may prove satisfactory, there still remains the problem of its experimental demonstration. The determination of whether or not synergism exists in a specific situation may be influenced by the definition of synergism employed. Also, the interpretation of results obtained may be modified by the mechanism the investigator may believe responsible for the concerted effective action of the drugs.

Under definition 3 above, synergism would exist if the combined action of the agents represented simply the sum of their separate actions. For the investigator who used agents in conjunction simply in order to get the advantage of their separate effects, the simple summation of separate actions would also represent synergism. This is in contradiction to the requirements of synergism under several of the other definitions above. Definitions 1 and 2, for example, require that the combined effect be greater than the sum of separate effects. An alternative test employed for synergism depends on the comparison of the potency of the mixture with the sum of the potencies of the components. Under this test, if the components were not synergistic but were essentially different concentrations of the same effective substance, the mixture would elicit the same response as a single level of either drug alone containing the equivalent amount of effective substance. This test may be applied graphically (14, 30, 36, 37). The amounts of either drug alone required to elicit a specified end-point are determined. Similar determinations are made for mixtures of the drugs in various proportions. These determinations are plotted arithmetically with the level of one drug shown as abscissa, the other as ordinate. If the determinations fall below the line connecting the levels required of either drug alone, it is considered a demonstration of synergism; if they fall above the line, it is considered to demonstrate antagonism; while results falling on the line indicate simple additivity of dose.

Logically, this scheme is applicable for drugs with similar actions and having parallel logarithmic dose-response curves (16).

Essentially, definitions 1, 2, 4, and 5 require that in synergism the combined effect of two drugs should be greater than could be predicted from their separate effects. The more common rules employed for predicting combined effect from separate effects are that either the effects themselves be additive or that the doses be additive.

The notion that the existence of synergism should hinge on the nonpredictability of the joint effect is somewhat weakening. It is evident, as pointed out in the previous section, that the extent of effect of a combination of drugs on a biological system is merely a reflection of the manner in which biochemical mechanisms are influenced. Nonpredictability could reflect a defective prediction model or an incomplete knowledge of the separate effects of the drugs. For example, the case may be considered in which either drug alone is ineffective in curing disease because of the presence of resisters, whereas the combination is effective because of the small likelihood that any organisms will be resistant simultaneously to both drugs. Under the usual procedures for predicting joint effect, this result would be taken to indicate synergism. However, if the prediction model incorporated a biochemical understanding of the separate effects of the drugs on the organisms (in addition to knowledge of the cure rates attained with each drug) the high cure rate for the combination might well have been predicted. The observed joint effect would then seem to be no more than the sum of the separate effects and would no longer be considered a demonstration of synergism.

From a biochemical point of view, the criterion of predictability is no longer necessary. It would appear to be more useful to investigate synergism in molecular terms, with reference to biochemical mechanism, as discussed above. From this point of view it is possible to examine for synergism, at various levels of interest, in terms of "molecule (concentration)-response." Thus: (a) Does the combination of drugs result in an increase in response for a given number of molecules, for either or both drugs? (b) Is the increase in response such that it cannot be duplicated by any number of molecules of either drug alone? (c) What are the alterations in two (or more) responses, and what are their interrelationships? Is there an increase in the response of interest, relative to a limiting response? This would apply to the chemotherapeutic case, where, for example, an increase in an antibacterial response is limited by an increase in a
toxic response for the host. Inherent in each of these types of synergism is a cooperation between the agents in achieving their joint effect, with only the nature of the cooperation differing. Synergisms could then be classed as explained or unexplained according to whether or not the state of knowledge is adequate to account for the observed effects.

**Therapeutic Synergism**

As has been indicated above, therapeutic synergism indicates a situation in which combination treatment results in an improved therapy. The practical application of such a definition requires appropriate methods for identifying improved therapies.

The response to treatment is obviously complex, encompassing not only the curative aspects of the therapy, but also all the side effects, including toxicity and lethality. Theoretically, the entire spectrum of responses should be taken into account in a determination of the relative merits of alternative treatments.

In practice, however, it is frequently possible, in a given therapeutic situation, to restrict the consideration to a limited number of effects of treatment. Thus, where treatment is frequently unsuccessful, modifications of therapy may be sought which increase the relative frequency of cures. Where treatment is ordinarily successful, an improved therapy might be one which resulted in earlier cures, or which eliminated toxic side-effects of treatment. Where treatment does not ordinarily lead to cures, an improved therapy could be one which simply increased the survival time of the host. Thus, it is the deficiencies of the current therapy which provide a basis for the determination of improvement in therapy. Improvement in therapy by combination treatment may serve, in the practical application of such a definition requires appropriate methods for identifying improved therapies.

**Combination Chemotherapy of Experimental Neoplasia From the Viewpoint of Therapeutic Synergism**

In general, the concept of "therapeutic synergism" has received little attention in the various studies of the effectiveness of drug combinations against experimental tumors. To the extent that this concept has played some role in the investigations conducted in this laboratory, a brief account of these studies may serve as a frame of reference for a review of other investigations. These examples should be considered as illustrative rather than comprehensive. The investigation of combination chemotherapy was part of a general program designed to investigate factors which may influence the effectiveness of chemotherapeutic agents, taking into account the host-tumor-drug relationship. It was observed that the antileukemic specificity of action of a drug may be altered by factors such as: the time when treatment is initiated; the number of leukemic cells; schedule of treatment; dietary intake, age and weight of the host, etc. (19, 24-27, 31).

In these investigations attention was focused on the effect of therapy on the survival-time of the leukemic host. A reduction in the size of the local tumor at the site of leukemic inoculation or suppression of peripheral blood leukocytosis, although noted, was not considered a sufficient requirement for improved therapy unless there was an accompanying extension in the survival time of the animal. In all these studies, the toxicity of the drug for the host was a prime limiting factor to the achievement of more effective therapies.

As conducted, some of the studies permitted a temporal separation of early deaths resulting from drug toxicity from late deaths attributable to the progressive growth of the tumor (22). This temporal separation permitted an examination of the relationship between the toxicity of a level of treatment and the average survival time for animals succumbing to tumor growth. A criterion was thus established for determining which factors increased and which diminished the effectiveness of chemotherapy. Of the alternative procedures, one for which survivors of drug toxicity showed a higher average survival time at a given level of toxic mortality would be deemed superior. It is evident that this criterion could also be used for comparing the effectiveness of combination chemotherapy with that of single drug therapy, thus establishing a procedure for ascertaining the existence of therapeutic synergism.

In other studies, in which it was not practicable to separate drug deaths from tumor deaths, a different criterion for comparing the effectiveness of alternative therapies was employed. The principle of this criterion was that corresponding to any particular therapy there was a level of treatment which elicited a maximum average survival time for deaths from any cause. At lower treatment levels deaths occurred earlier as the result of more progressive tumor growth, while at higher levels...
deaths occurred earlier because of increased toxicity. This criterion, too, could be used in determining therapeutic synergism (32).

Perhaps a more desirable criterion for establishing improved therapy would be based on the proportion of animals kept alive and tumor-free by virtue of it. There has been the experience in this laboratory, however, that a sharply reduced percentage of “tumor takes” could be demonstrated readily by the simple expedient of employing a low tumor inoculum at a threshold level for 100 per cent mortality from tumor. Such a tumor challenge was believed too weak, however, to select out only those therapies having relatively important antineoplastic properties. Instead, typically, the studies were conducted at tumor-inoculum levels on the order of 100 times that yielding 100 per cent tumor takes in the titration of tumor cell potency (28). Also, as more effective procedures were developed for increasing the survival time of the tumor-bearing host, the challenge was strengthened still further by delaying the initiation of treatment until only 2 or 3 days before the time at which the mice would otherwise have died (32, 77).

With the advent of improved therapeutic techniques, reduction in the percentage of “tumor takes,” or “cures,” could become a routine criterion for comparing alternative therapies. At the present time, multiple treatments with A-methopterin initiated early does permit a substantial reduction in percentage “takes,” even when the leukemic inoculum is relatively high (31). No similar success has yet been achieved in the treatment of advanced leukemia.

Some examples of the application of these criteria in investigations of combination therapy of leukemia L1210 are presented below.

**Aminopterin and citrovorum factor.**—The initial investigation of the combination treatment of leukemia L1210 with aminopterin and citrovorum factor was prompted by the fact that the toxic lethality of aminopterin for the host limited its effectiveness as an antileukemic agent. If citrovorum factor served to reduce the toxic effect of aminopterin without reducing its antileukemic effect, the combination treatment would be more effective. This, however, did not prove to be the case. Instead, while citrovorum factor did reduce toxicity, it reduced the antileukemic effect to a proportionately greater extent, and the combination treatment was less effective than treatment with aminopterin alone. Thus, concomitantly administered citrovorum factor, in a sense, antagonized the therapeutic effectiveness of aminopterin (22, 23). A similar result was obtained when folic acid was administered 1 hour prior to aminopterin (22).

Offhand, such a result would suggest that the leukemic cells were more efficient than those of the host in utilizing citrovorum factor to offset aminopterin toxicity. An alternative explanation was that the leukemic cells had proportionately less metabolite and were thereby more sensitive to aminopterin. The immediate availability of citrovorum factor would then be of more benefit to the tumor than the host. To discriminate between these alternatives, an experiment was performed in which administration of citrovorum factor was delayed for 12 and 24 hours following aminopterin treatment (23). Charts 1 and 2 show some of the results obtained in the experiments performed. Chart 1 shows that, for a given level of toxic mortality, concomitant administration of citrovorum factor increased survival time, while concomitantly administered citrovorum factor diminished survival time, as compared with aminopterin alone (no CF) (22).
protection to the host; but the antileukemic action was apparently complete, and the citrovorum factor could no longer protect the tumor. This permitted the employment of higher doses of aminopterin, and the net result was an enhanced therapy. The line for concomitant citrovorum factor is well below the others, reflecting the protection against toxicity afforded to the tumor.

This demonstration of how delayed citrovorum factor synergizes the therapeutic effectiveness of aminopterin is of special interest. It illustrates that in therapeutic synergism the usual rules for looking for synergism may go by the board. Essentially, citrovorum factor is an antagonist of the antitumor effectiveness of aminopterin, and routine methods for testing the drugs in combination would not have revealed its capacity for improving therapy. A simple change in schedule has converted an antagonist to a synergist.

Another study (27) showed that delayed citrovorum factor increased the antileukemic action on repeated administration of aminopterin (previous results were obtained with single treatment). There was some indication that with multiple treatment it was important to have a long enough interval between treatments so that the tumor would not receive protection from residual amounts of previously administered citrovorum factor. The lesser frequency of treatment advisable when delayed citrovorum factor is employed could, in some instances, offset the benefits obtained from the higher dose levels of aminopterin which the delayed citrovorum factor makes possible.

It was reasonable to expect that delayed administration of citrovorum factor would modify the effectiveness of treatment with A-methopterin in the same manner as with aminopterin. Indeed, the delayed administration of citrovorum factor provided somewhat more extensive antileukemic action with A-methopterin as well as with aminopterin (26). This report also confirms an earlier observation (29) that A-methopterin is more effective than aminopterin against this leukemia (L1210) when the drugs are evaluated on the basis of the effectiveness of the therapy rather than on the basis of the amount of antitumor damage obtained with a fixed amount of material.

6-Mercaptopurine plus adenylic acid.—The use of these drugs in combination had a motivation similar to that for the aminopterin-citrovorum factor combination. The lethal toxicity of 6-mercaptopurine was the limiting factor in the effectiveness of that drug as an antileukemic agent, and adenylic acid could reduce this toxicity (18). Goldin et al. (28) report results obtained when adenylic acid was administered to leukemic mice concomitantly with, or 1 hour following, single administration of 6-mercaptopurine. When administered at either time, adenylic acid reduced the lethal toxicity of 6-mercaptopurine, but this reduction in toxicity was accompanied by a reduction in the survival time of mice succumbing to tumor. The protection by adenylic acid against the toxicity of 6-mercaptopurine was essentially the same for both tumor and host, so that the relationship between survival time and toxic mortality was virtually unaltered.

A-methopterin plus 6-mercaptopurine vs. early tumor.—Essentially, the initial investigation of the antileukemic action of these drugs in combination was conducted as a trial attempt at using improvement in therapy as a criterion for evaluating the effectiveness of combinations of antineoplastic agents. Reports in the literature with such combinations almost invariably indicated that they resulted in increased antineoplastic activity. Ordinarily, no account was taken of the fact that the combination also resulted in increased toxicity for the host. The experiment performed was designed to permit the taking into account of both host toxicity and antitumor effect in the evaluation of therapy.

An important consideration was the relative...
amounts in which the drugs were to be administered. If the drugs in combination could provide a superior therapy, this might be evident in only a specific range of relative proportions. In other proportions, however, the therapeutic efficacy of the combination could lie between, or possibly even below, the efficacies of the separate drugs. For this reason, the study was conducted employing a range of relative proportions in addition to each drug separately (30).

Chart 3 summarizes the results of this investigation in which the drugs, alone and in combination, were administered as single treatments. For each of the proportions employed, it shows the relationship between the average survival time of mice succumbing to tumor and the lethal toxicity of the dosage administered. There was no indication that any of the drug combinations provided a therapy superior to that obtained with A-methopterin alone. The results also demonstrated the superior therapy provided by A-methopterin over that for 6-mercaptopurine. Although the usual methods of analysis, where host toxicity is not taken into account, suggested some synergism, this was counterbalanced by a corresponding synergism in the toxic effect for the host (30).

This study emphasizes the caution with which the usual demonstrations of synergism, or even its absence, must be interpreted. A reported antineoplastic synergism may have no carry-over in therapy. Also, failure to find synergism for drugs having qualitatively similar effects could reflect failure to use the drugs in optimal proportions. If the efficacy of a combination is a continuous function of the relative proportions in which the drugs are employed, it necessarily follows that in some range of proportions, whether or not the drugs are synergistic, the efficacy of the combination will lie between the efficacies of the separate drugs.

A-methopterin plus 6-mercaptopurine vs. advanced leukemia.—A limitation of the foregoing demonstration of absence of therapeutic synergism between A-methopterin and 6-mercaptopurine was that it referred only to single administration and not to repeated treatment.

An experiment was, therefore, performed with the repeated administration of the drugs in combination (77). The experiment as performed was not comprehensive in the sense of employing the drugs in a range of proportions. Rather, because of the necessity for frequently repeated treatment, only a single relative proportion was selected. There was, nevertheless, the opportunity for the combination to prove superior to the separate drugs.

At the time this experiment was performed, emphasis had shifted to treatment of advanced leukemia. Rather extensive increases in the survival times of leukemic mice had already been demonstrated when repeated treatment was initiated early (26, 32). Advanced leukemia was accordingly employed in the current investigation, and, further, daily doses were administered, since this interval of treatment had been shown to be optimal for advanced leukemia (32, 77). The experiment was augmented by groups in which A-methopterin was administered every 3 days—alone, and in combination with daily doses of 6-mercaptopurine.

![Chart 3](chart3.png)

**Chart 3.**—Effect of treatment of early leukemia (L1210) with A-methopterin and 6-mercaptopurine on the survival time-toxic mortality relationship. At equal toxicity for the host (expressed in probits), the increase in survival time with A-methopterin alone was not exceeded by any of the combinations (29).

As performed, the experiment did not permit a separation of toxic and tumor deaths. Instead, a different criterion for comparing alternative therapies was employed; the therapies were judged on the basis of the maximum median (or average) survival time achieved, irrespective of the dose level or the cause of death. Chart 4 summarizes the results obtained (77).

Examination of the left panel of Chart 4 shows that the maximum median survival time for A-methopterin alone was greater than that for either 6-mercaptopurine alone or for the drugs in combination. Thus, in the proportion used, there is no indication of synergism. The superiority of A-methopterin over 6-mercaptopurine is again demonstrated.

The right panel of Chart 4 shows an interesting contrast. Here, the combination is superior to the separate drugs; but the combination still does not
match the results obtained with daily A-methopterin alone as shown in the left panel. The impression of therapeutic synergism indicated by the right panel vanishes when it is considered that the optimal treatment indicated by the experiment as a whole still involves the daily administration of A-methopterin.

The superiority of the combination over separate drugs in the right panel is readily comprehensible. For advanced leukemia, treatment with A-methopterin every 3 days is a nonoptimal schedule (32). In the combination, the daily treatment with 6-mercaptopurine serves to shorten the time interval between antileukemic treatments, resulting in an improvement over the results obtained with A-methopterin alone every 3 days (77).

This is an example of how scheduling effects can create false impressions of synergism. The existence of optimal schedules always leaves open the possibility of such “pseudo-synergism.” For example, if a daily schedule is optimal for two essentially identical drugs, a combination treatment in which the drugs are alternated daily will be superior to either drug alone administered every 2d day. Alternatively, if treatment every 2 days is optimal, such a combination treatment will be inferior to single drug treatment every 2d day, giving the appearance of antagonism.

Thioquanine or 6-mercaptopurine plus azaserine vs. advanced leukemia.—Charts 5 and 6 show results obtained with the indicated drugs in combination and separately (38). The experiment was essentially similar to that described above with A-methopterin and 6-mercaptopurine, and the criterion used in judging the effectiveness of drug combinations is the same. The drugs, alone and in combination, were administered daily against advanced leukemia (L1210).

Examination of the charts shows that, while azaserine increased the effectiveness of low levels of 6-mercaptopurine or thioguanine, it did not raise the median survival time beyond that obtained with sufficiently high levels of 6-mercaptopurine or thioguanine alone. There is thus no indication of therapeutic synergism.

An interesting point is brought out by these results. It may be noted, for instance, that the lowest level of 6-mercaptopurine and azaserine employed in combination produces a distinct increase in survival time. This occurs even though the individual drugs are completely ineffective at these low doses. These doses, it should be noted, are of the order of only 15–20 per cent of the
maximally effective doses of the individual drugs. Ordinarily, results such as these, where a combination of individually ineffective doses proves to be effective, would be taken as evidence of synergism. The present experiment shows that such an apparent demonstration of synergism does not necessarily indicate that the drugs in combination are superior to the better drug alone.

REVIEW OF INVESTIGATIONS OF COMBINATION CHEMOTHERAPY OF EXPERIMENTAL NEOPLASIA

The Addendum gives a brief description of 24 published reports of investigations involving the combination chemotherapy of experimental tumors. The descriptions are limited in nature and omit mention of many valuable aspects of the investigations.

For each report the table shows the compounds and experimental tumors employed. Also indicated is the motivation leading to the employment of the drugs in combination. This includes any stated mechanism which could result in agents in combination being effective and/or the reason for selecting the particular agents tested. In some cases agents are indicated to have been tested in combination purely on a trial basis.

Under “Criterion for Synergism or Potentiation,” the Addendum shows any principle stated as employed, or apparently employed, by the investigator in judging whether or not synergism or potentiation has occurred or for judging whether or not combination therapy is superior. Some reports present data for comparison only, and no judgments as to the relative effectiveness of combinations are made. The measure of antitumor effect of treatment, usually tumor growth (weight) or average survival time, is also indicated.

The “Summary and Comment” of the Addendum indicates whether or not the investigation resulted in a finding of synergism or potentiation in accordance with the investigator’s criterion for adjudging combinations to be superior. Generally, also, it includes such comments as the reviewers have deemed to be relevant. In most cases these comments point out factors which may restrict the nature of the demonstrated effectiveness of combination therapy.

GENERAL REVIEW AND CRITIQUE

The most frequently cited reason for expecting agents in combination to be effective is that they can result in damaging the tumor simultaneously via several mechanisms. This can result from the sequential or concurrent blockade of metabolic pathways. The expectation that the increased tumor damage will not be accompanied by increased toxicity for the host is frequently stated.
The problem of the resistance of tumor cells is also cited as justifying the use of drug combinations. The possibility that an ineffective agent may augment the antitumor effect of a drug on one hand or diminish drug toxicity for the host on the other are also considered as leading to effective combinations. In two cases the basis given for using agents in combination with 8-azaguanine is that they lead to potentiation by virtue of their inhibition of 8-azaguanine deamination. This type of prolongation effect could correspond to Veldstra's model of competition for sites of loss (76).

The bases for selecting particular compounds for trial are varied. One frequently given is that the compound is an analog or inhibitor of a metabolite for which the tumor concentration is low. This justification stems from the concept for achieving antitumor effects by taking advantage of differential concentrations of metabolite in tumor and host (1, 54, 62, 63). There is, of course, a calculated risk in applying this concept. The concentration may be low if the metabolite is not particularly essential for the tumor. Other difficulties arise in applying the concept. The low concentration of a metabolite in the tumor may be more apparent than real, reflecting high water content of tumor (48), or possibly even reflecting low concentration in necrotic, nonviable portions of the tumor. Moreover, the tolerance of the host for the drug may be limited by a still lower metabolite concentration obtaining in a specific vital tissue of the host.

The principal criterion employed for judging the merits of combination therapy was that the drugs in combination elicit greater antitumor effect, as reflected in reduced tumor weight or extended survival time for the host, than they do singly. Occasionally, the indicated criterion is that the response to the drugs in combination be more than additive effectwise. Another occasionally used criterion is that combinations of fractions of maximum tolerated levels of the individual drugs be more effective than the drugs individually at their respective maximum tolerated levels. Essentially, this criterion is that the response to the drugs in combination be more than additive dosewise.

The criterion that the combination be more effective than the separate drugs is in general untenable when both drugs are active. In combination, the level of treatment is, effectively, higher. However, in applying the criterion, the investigators frequently point out that one or the other, or sometimes both, of the drugs alone is ineffective. In such case, the criterion of greater effect for the combination is essentially one in which the drugs are more than additive effectwise or dosewise. The principal weakness in applying this criterion lies in the failure to demonstrate properly the ineffectiveness of individual drugs. Generally, instead of showing that the drug is ineffective over a wide range of dose levels, it is demonstrated that only particular levels of the drug are ineffective. Use of individually ineffective levels of drugs in combination may prove effective simply because the level of treatment has been raised. Another invalid procedure employed for proving a drug to be individually ineffective was to demonstrate that at the level administered it did not achieve a statistically significant antitumor effect. Thus, even when an antitumor effect for a drug appears, but is not significant in each of several repeated trials, the drug may, improperly, be judged as ineffective.

What role does the toxic effect of the drug combination for the host play in these investigations? In some cases it is quite clear that the possibility that, in combination, toxicity for the host will be altered is of main concern. It was, in fact, of chief concern in the case in which an antibiotic was used together with the tumor inhibitor (8). The expressed appreciation of the problem of combined host toxicity has, in general, however, not led to investigations conducted in such a way as to take it fully into account in judging the effectiveness of drug combinations. To do so would generally have required a wider range of doses of the drugs alone and in combination than was generally employed. There was, nevertheless, underlying these investigations, an awareness of the need for demonstrating therapeutic synergism.

Careful reading of the reports indicates that this awareness is somewhat universal. The problem simply is that it is not reflected in the design of the investigations as conducted. Frequently, in the reports, it is indicated that the drugs in combination were expected to achieve increased tumor damage without important additive toxic effects for the host. The investigations as performed and analyzed, however, usually ignore the problem of increased host toxicity. The employment of drugs in combination at or below the separate maximum tolerated levels is assumed to take care of the problem. It would, if the host toxicities were not additive; but if the possibility is open for synergic antitumor damage, why not also for synergic host toxicity? In some cases the drugs in combination are found to have little host toxicity, while achieving extended tumor damage. This would tend to occur when the separate drug levels are relatively ineffective and leaves open the possibility for matching the drugs in combination by altering treatment levels for the separate drugs.

Occasionally, the reverse situation may occur. The drugs in combination are found to be less ef-
fective, as indicated by average survival time, than separately. This could occur when the augmented host toxicity of the combination is especially serious. Lower levels of the drugs in combination and altered proportions in the combination should be considered.

The underlying weakness in the reported investigations of combination chemotherapy is the less than comprehensive way in which they are conducted. Although no investigation can be wholly comprehensive, they have not been complete even within the framework in which they were performed. The employment of relatively thorough titrations of the drugs separately and in combination in attempts at ascertaining the nature of joint drug action would be of prime importance in this regard. Experimentation performed with the requirement of relatively thorough titration fulfilled would provide a somewhat firmer basis for establishing the existence of synergistic effects of interest to the investigator.

CONCLUSIONS

The present status of research in the treatment of experimental neoplasia with combinations of chemical agents has been reviewed. Fundamental advances have been made in this field, particularly in the development of biochemical concepts pertaining to mechanisms underlying the problems of synergism and potentiation. The dilemma resulting from the use of various definitions of synergism and potentiation and from the requirement that synergism implies nonconformity to prediction models of joint action is resolved on consideration of biochemical mechanism. The biochemical approach substitutes, for predictability, a description of mechanisms which may result in cooperative action of drugs in terms of enhanced responses of interest. Such cooperation may be examined with respect to the host, the tumor, or the host-tumor relationship.

There are two major obstacles impeding the application of the theoretical biochemical approach to the biological problem of synergism. (a) How are appropriate combinations of agents to be selected? The proper choice of agents in combination therapy requires additional detailed knowledge of in vivo metabolic transformations. Without such knowledge the choice of agents must, perforce, remain largely empirical. (b) Is the observable response an appropriate measure of enhanced activity, and does it, indeed, reflect the implicated biochemical mechanism? For this purpose, wholly quantitative procedures are required for the evaluation of dose-response relationships with respect to the host, tumor, and host-tumor relationship. Methods of inhibition analysis, extended to the mammalian organism, may provide a useful tool in integrating the biochemical and biological approach.

The concept of “therapeutic synergism” was stressed, particularly in view of its potential applicability to therapy.

ADDENDUM

SUMMARY OF INVESTIGATIONS OF COMBINATION TUMOR CHEMOTHERAPY

**Investigators:** Barvick and Goodson (8)

**Tumors:** Sarcoma 180

**Agents employed:**
- A-methopterin
- N,N-bis(2-chloroethyl)aniline
- Triethylene melamine
- Erythromycin

Motivation: Authors indicate that the biochemical concepts of “antimetabolites,” “sequential blocking,” and “simultaneous blocking of alternate pathways” are useful in the selection of compounds for combination therapy. Here, presumably, they have led to the selection of an antimetabolite, two alkylating agents, and an antibiotic.

Criterion: Agents are tested at maximally tolerated levels. In the A-methopterin-triethylene melamine combination, they are also tested at fractions of these levels. The various treatments are compared on the basis of tumor area and the survival time and weight loss of the host.

Summary and Comment: The authors note that, while each of the combinations used reduced tumor growth and increased survival time, they were accompanied by undesirable side effects for the host. There are indications in the data that the increased effect of combinations could have been matched by modifying levels of separate drugs. An attempt at getting at nonspecific effects of reduction in food intake was unsuccessful.
INVESTIGATORS; TUMORS; AGENTS EMPLOYED

**Motivation:** Treatment of tumor with combinations of B₆ antagonists and acid hydrazides was suggested by the observation of their synergistic inhibition of growth of E. coli. Employment of a pyridoxine-deficient diet in conjunction with the combinations was suggested by the observation that deoxypyridoxine hydrochloride caused regression of a lymphosarcoma in mice on such a restricted diet, and also by the low pyridoxine levels in Carcinoma 755.

**Criterion:** Effects of drugs in combination and separately on tumor growth are compared, their toxic effects for the host as indicated by weight loss being taken into consideration. Generally, a range of dose levels was employed.

**Summary and Comment:** In general, the results show the acid hydrazides to potentiate tumor inhibition by B₆ antagonists when administered to animals on a B₆ deficient diet. Potentiation is not observed for animals on a complete diet. The combined inhibitory action of acid hydrazides and B₆ antagonists for the tumor in animals on the deficient diet is reversed by administration of vitamins of the B₆ group. These results seem to apply even after allowing for altered toxicity of the agents in combination. To the extent that a range of doses was employed, it is possible to make allowances for altered toxicity and mortality. Animals are sacrificed on the 8th day following tumor implantation, and only 1 day after their last treatment. Toxic deaths that might have occurred later are not reflected in the mortality data shown. The weight change of untreated animals is not reported.

In conclusion, these results suggest that the combination of acid hydrazides and B₆ antagonists may offer a promising approach for the treatment of tumors in animals on a B₆ deficient diet. Further studies are needed to confirm these findings and to explore the clinical potential of this approach.
**SEPTEMBER 10,** 1957

**GOLDIN AND MANTEL—Combination Chemotherapy of Neoplasia: Review**

**SYNOPSIS:**

- **Motivation:** The use of combinations of agents antagonistic to the metabolism of methyl groups suggested by results implicating methyl groups in the formation of leukocytes.

- **Criterion:** Combinations of agents are required to yield a greater extension in survival time than separate drugs provide.

- **Summary and Comment:** The drugs in combination, at individually ineffective levels, proved to be effective. At higher doses, the drugs were more effective in combination than singly, but there was less than an additive effect. The investigators refer to the effects obtained at the lower levels as synergistic. Similar effects, however, can be obtained by subdividing an effective level of a single drug into ineffective aliquots and then reconvening. See current Charts 5 and 6 as examples where similar results were noted as in this study, but where the combination was still not optimal.

- **Motivation:** Development of tumor by drug resistance leads logically to the combined use of drugs. Combined use of agents differing in their modes of action should prove still more effective. The agent used here with A-methopterin was one of a class, pyrimidines, which had been found to inhibit Sarcoma 180 and Leukemia AK4.

- **Criterion:** Combination treatment is required to result in a greater than additive effect. More particularly, combinations of drugs at levels individually ineffective in extending survival time should be effective. Some effects on tumor volume are also noted.

- **Summary and Comment:** The drugs in combination, at individually ineffective levels, proved to be effective. At higher doses, the drugs were more effective in combination than singly, but there was less than an additive effect. The investigators refer to the effects obtained at the lower levels as synergistic. Similar effects, however, can be obtained by subdividing an effective level of a single drug into ineffective aliquots and then reconvening. See current Charts 5 and 6 as examples where similar results were noted as in this study, but where the combination was still not optimal.

- **Motivation:** Catalytic role of hormones in biochemical reactions suggests possibility for their increasing antitumor effect.

- **Criterion:** Addition of hormone required to increase the inhibition of tumor growth provided by 8-azaguanine.

- **Summary and Comment:** Antitumor activity of 8-azaguanine was increased by hormones, especially by diethylstilbestrol. Apparent increase in toxicity is ignored. Diethylstilbestrol alone was found also to be an inhibitor of tumor, but levels employed produced weight loss and death for the host.

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**INVESTIGATORS; TUMORS; AGENTS EmployED**

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Tumors</th>
<th>Agents employed</th>
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<tbody>
<tr>
<td>Goldin et al. (21)</td>
<td>L1210</td>
<td>8-Azaguanine, Aminopterin, Alpha-pelatinate, Triethylene melamine, Nitrogen mustard (HN2)</td>
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<tr>
<td>Law (45)</td>
<td>Leukemia L1210</td>
<td>8-Azaguanine, Aminopterin, Triethylene melamine, Alpha-pelatinate</td>
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<tr>
<td>Morrison and Higgins (49)</td>
<td>Leukemia L1210</td>
<td>A-methopterin, N-Methylformamide, Formamide, Ethionine, Triethylcholine</td>
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<tr>
<td>Nadel and Greenberg (50)</td>
<td>Leukemia L1210</td>
<td>A-methopterin, 2,4-Diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine</td>
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<tr>
<td>Shapiro (56)</td>
<td>Carcinoma 755</td>
<td>8-Azaguanine, Testosterone, Diethylstilbestrol</td>
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**ADDITIONUM—Continued**

**Motivation:** Combination treatment of tumors could prove more effective provided the systemic toxicity for the host was not sufficiently additive to interfere with the augmented antineoplastic effect.

**Criterion:** When dose levels are employed within the effective range, drugs in combination are required to achieve a greater effect than they do separately. Effects noted are: average survival time, tumor volume, white blood count. Animal weight change is also observed. The effective range is defined as the range of dosage within which the average survival time can still be increased by higher levels of treatment. Beyond this range the average survival time is diminished by increased dosage because of enhanced toxicity.

**Summary and Comment:** Combination treatment generally resulted in increased tumor inhibition and greater reduction in white blood counts. This was not invariably reflected, however, in an extension in the survival times of the animals. That seems to depend in large measure on whether or not the level of treatment employed is within the effective range for the combination. The intention of the investigators was evidently to determine whether or not combination treatment could result in improved therapy, but the experiments performed were not completely appropriate for the purpose. The apparent presence or absence of synergism depended on the treatment levels employed.

**Motivation:** Use of combinations of agents antagonistic to the metabolism of methyl groups suggested by results implicating methyl groups in the formation of leukocytes.

**Criterion:** Combinations of agents increased survival time. It is suggested by the authors that, in combination, potent tumor-inhibiting qualities can be retained while toxic effects are avoided. The results obtained can be readily attributed to the effectively higher dosage level in the combination treatment. It is not clear how the investigators would handle the case of an animal dying late without evidence of leukemia. The death may be attributable to toxicity, but it also represents evidence of high antitumor effect.

**Motivation:** Single-drug treatment is ineffective because of development of resistance by tumor. This possibility is minimized by combination treatment.

**Criterion:** Combination required to yield a greater extension in survival time than separate drugs provide.

**Summary and Comment:** 8-azaguanine and A-methopterin were found to be potentiating. However, levels of drug employed were well tolerated by the mice, indicating the possibility that the survival time for the combination could have been matched by raising the level of single-drug treatment.
MOTIVATION; CRITERION FOR SYNERGISM OR POTENTIATION; SUMMARY AND COMMENT

ADDENDUM—Continued

<table>
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<tr>
<th>INVESTIGATORS; TUMORS; AGENTS EMPLOYED</th>
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<th>CRITERION</th>
<th>SUMMARY AND COMMENT</th>
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<tr>
<td>Shapiro and Fugmann (59)</td>
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<td>Carcinoma 755</td>
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<td>Folic acid</td>
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<td>Riboflavin</td>
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<td>Folic acid</td>
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<td>7-Methyl folic acid</td>
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<td>Vitamin B1</td>
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<td>Shapiro et al. (57)</td>
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<td>Carcinoma 755</td>
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<td>8-Azaguanine</td>
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<td>Deoxypyridoxine</td>
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<td>Testosterone</td>
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<tr>
<td>6,7-Dimethyl-9-hydroxy-ethyl-isoalloxazine (U-2113)</td>
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<tr>
<td>Shapiro et al. (62)</td>
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<td>Carcinoma 755</td>
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<tr>
<td>Deoxypyridoxine</td>
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Motivation: Combinations of drugs may result in greater antitumor effect by inflicting simultaneous damage on different metabolic pathways. Use of the riboflavin analog, flavin, in conjunction with 8-azaguanine is suggested by the low riboflavin concentration of tumor.

Criterion: Addition of flavotin is required to increase the inhibition of tumor growth provided by 8-azaguanine.

Summary and Comment: Prior administration of flavotin is found to increase significantly the tumor growth inhibition of 8-azaguanine in female mice. This effect is reversed by riboflavin. The authors report no antitumor effect for flavotin alone in either sex, and no augmented effect for flavotin, added to 8-azaguanine, in male mice. Their data actually show a consistent antitumor effect for flavotin in these cases, but in no single experiment is it significant. The combination treatment frequently results in increased mortality, the added mortality being greater in males.

Motivation: Chemical combinations may affect tumor growth adversely, to the point of eradication, without producing either serious host toxicity or drug resistance. In the present instance the drugs in combination may have a sequential blockade effect.

Criterion: The authors state that synergism is indicated when the combined effect of two agents is greater than a simple algebraic summation of their individual carcinostatic activities.

Summary and Comment: The drugs in combination lead to significant reduction in tumor weight more frequently than does the pyrimidine alone. Whether or not this reduction is greater than the sum of the separate carcinostatic effects of the agents is not clear, since ethionine was generally not tested by itself in each experiment. It is apparently assumed that ethionine in itself is not carcinostatic. The authors considered their data from experiments in which ethionine was tested alone as indicating that it was not carcinostatic, since it produced a significant tumor weight reduction in only one of seven experiments. Their data, however, indicate that ethionine produced a relatively consistent though not significant reduction in tumor weight.

Motivation: Combination therapy was suggested by the observation that with bacteria such treatment overcomes the problem of "drug fastness." For selected diseases combination therapy attacks metabolic functions simultaneously at several points. Use of the pyridoxine antagonist, deoxypyridoxine, with 8-azaguanine was suggested by the low concentration of pyridoxine in tumor. 7-Methyl folic acid was employed, since it is a vitamin analog. Folic acid and vitamin B12 were employed with the anticipation that they would inhibit the antitumor effect of 8-azaguanine.

Criterion: Inhibition of tumor growth of drugs in combination is compared with that of separate drugs.

Summary and Comment: There are indications in the data presented that the antitumor effect of 8-azaguanine was enhanced by each of the added agents, but the enhanced effect does not show up consistently for some of the agents. The possibility of antitumor effect for the agents alone is not clearly ruled out in every case. Levels of folic acid employed alone were lethal for male mice, but the lethality was reduced by adding 8-azaguanine. This may be an example of the roles of metabolite and antagonist being reversed when the level of treatment with metabolite is excessive.

Motivation: Low concentration of riboflavin in tumor suggested that addition of an antimetaboloid (U-2113) would increase antitumor effect.

Criterion: Combination required to inhibit tumor growth more than separate drugs.

Summary and Comment: The quadruple combination was found to have greater carcinostatic effect. Increased carcinostasis was achieved by adding doses of deoxypyridoxine and U-2113 which by themselves were nontoxic for both host and tumor. Authors ignore indications in their data that the quadruple combination has increased toxicity for the host.

Motivation: Low concentration of pyridoxine in tumor suggests that addition of a pyridoxine inhibitor (deoxypyridoxine) would increase antitumor effect.

Criterion: Addition of pyridoxine inhibitor required to increase the inhibition of tumor growth provided by other antimetabolite combinations.

Summary and Comment: Antitumor activity of 8-azaguanine plus testosterone was increased by the addition of deoxypyridoxine. This, however, resulted also in increased mortality. Investigators suggest that it is likely that, if daily dosage administered were based on individual blood or tissue levels of pyridoxine, the increased mortality could have been avoided and the antitumor effect retained.
GOLDIN AND MANTEL—Combination Chemotherapy of Neoplasia: Review

ADDENDUM—Continued

<table>
<thead>
<tr>
<th>INVESTIGATORS; TUMORS; AGENTS EMPLOYED</th>
<th>MOTIVATION; CRITERION FOR SYNERGISM OR POTENTIATION; SUMMARY AND COMMENT</th>
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<tbody>
<tr>
<td><strong>Investigators:</strong> Shapiro et al. (63)</td>
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<tr>
<td><strong>Tumors:</strong> Carcinoma 755</td>
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<tr>
<td><strong>Agents employed:</strong> 6-Formylpteridine</td>
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<tr>
<td><strong>Summary and Comment:</strong> The quadruple combination showed the greatest carcinostasis, virtually stopping tumor growth. There is evidence of some toxicity, however, for the combination. In total, the quadruple combination employs a higher level of treatment than combinations of fewer drugs, suggesting the possibility of matching the quadruple combination result by raising the levels employed in other combinations.</td>
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</table>

**Investigators:** Shapiro et al. (61)

**Tumors:** Carcinoma 755

**Agents employed:** 8-Azaguanine

**Summary and Comment:** Prior administration of 6-formylpteridine was found to increase the inhibition of tumor growth provided by 8-azaguanine.

**Investigators:** Skipper (67)

**Tumors:** Chloro-Leukemia 1394

**Agents employed:** Urethan

**Summary and Comment:** The quadruple combination employs a higher level of treatment than combinations of fewer drugs, suggesting the possibility of matching the quadruple combination result by raising the levels employed in other combinations.

**Investigators:** Skipper (69)

**Tumors:** Carcinoma 755, E 0771

**Agents employed:** 6-Mercaptopurine

**Summary and Comment:** The quadruple combination employs a higher level of treatment than combinations of fewer drugs, suggesting the possibility of matching the quadruple combination result by raising the levels employed in other combinations.

**Investigators:** Skipper et al. (70)

**Tumors:** Leukemia AK4

**Agents employed:** Aminopterin

**Summary and Comment:** The approach here is essentially that of therapeutic synergism, but the need for evaluating agents alone and in combination over a range of doses was not considered. This could account for the general failure in the investigation to find synergistic combinations. There is confirmation of an earlier finding of synergism for the urethan-nitrogen mustard combination, but that combination is still less effective than the antifolics employed singly.

Motivation: Prior administration of 6-formylpteridine was found to increase the inhibition of tumor growth provided by 8-azaguanine.

Criterion: Addition of 6-formylpteridine required to increase the inhibition of tumor growth provided by 8-azaguanine.

Summary and Comment: Prior administration of 6-formylpteridine was found to increase the tumor growth inhibition of 8-azaguanine. No significant inhibition was found for 6-formylpteridine alone at the level employed.

Motivation: No specific motivation is given. It is stated to be a report of data that might be interpreted to indicate an antileukemic synergism between urethan and nitrogen mustard.

Criterion: The combination generally elicits an increase in life span and in the proportion of mice surviving 90 days. Data are presented on the toxic mortality of the drugs alone and in combination for normal mice. These are interpreted as indicating that they are not synergistic with respect to acute toxicity. Such lack of toxic synergism is not immediately obvious from the data. They do indicate, however, that the level of treatment with urethan alone could have been increased threefold without encountering significant toxic mortality. The possibility for matching the effectiveness of the combination by raising the level of treatment with the separate drugs is open.

Motivation: Combination treatment apparently was prompted by the reports of other groups that A-methopterin plus 8-azaguanine were effective in increasing life span of leukemic mice.

Criterion: Drugs in combination should be more effective in increasing life span than they are separately at the maximum tolerated dose.

Summary and Comment: According to the author his results suggest that 6-mercaptopurine plus A-methopterin may be somewhat more effective against L1210 than the separate agents. Azaserine plus either 6-mercaptopurine or 8-azaguanine are reported to be synergistic. The data presented suggest, however, that raising treatment levels for the separate drugs could have resulted in matching the effectiveness of the combination. Where the results do not indicate synergism or potentiation, this is stated, but data are not presented. Data on the tumoricidal action of 6-mercaptopurine plus 8-azaguanine are presented but not discussed.

Motivation: Attempt is to obtain combinations with synergistic antitumor effect but having little or no synergism with respect to host toxicity.

Criterion: Relatively nontoxic combinations are required to elicit greater extensions in survival time than nontoxic levels of the separate drugs.

Summary and Comment: The approach here is essentially that of therapeutic synergism, but the need for evaluating agents alone and in combination over a range of doses was not considered. This could account for the general failure in the investigation to find synergistic combinations. There is confirmation of an earlier finding of synergism for the urethan-nitrogen mustard combination, but that combination is still less effective than the antifolics employed singly.

Motivation: Low concentration of niacin in tumor suggests that addition of a niacin inhibitor (8-ethy1amino-1,3,4-thiadiazole) would increase antitumor effect.

Criterion: Addition of niacin inhibitor required to increase the inhibition of tumor growth provided by other antimetabolite combinations.

Summary and Comment: The quadruple combination showed the greatest carcinostasis, virtually stopping tumor growth. There is evidence of some toxicity, however, for the combination. In total, the quadruple combination employs a higher level of treatment than combinations of fewer drugs, suggesting the possibility of matching the quadruple combination result by raising the levels employed in other combinations.
**ADDITIONUM—Continued**

**Motivation:** Use of antimetabolites in combination can result in sequential and concurrent blockade of metabolic pathways. **Criterion:** Additions of antimetabolites required to further extend the survival time of leukemic mice. **Summary and Comment:** The triple combination—A-methopterin, 8-azaguanine, ethionine—was superior to individual drugs and dual combinations. Relatively low levels of the separate drugs were employed, and it is not clear what separate drugs at higher levels could have achieved. Ethionine is reported to prevent the potentiation of antileukemic activity of A-methopterin by ethionine. To what extent could increased toxicity for the host resulting from methionine treatment account for this result?

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Motivation: Combination treatment can result in the destruction of single drug-resistant variants. Also, combination treatment may result in the sequential blocking of a series of biochemical events.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumors:</td>
<td><strong>Criterion:</strong> Fractions of maximal tolerated doses of drugs in combination are required to inhibit tumor growth to a greater extent, or to increase survival time more, than do the maximal tolerated levels of the separate drugs alone.</td>
</tr>
<tr>
<td>Agents employed:</td>
<td><strong>Summary and Comment:</strong> Investigators appreciate that, generally, drugs in combination will seem more effective than separately if only because, effectively, treatment level has been increased. They try to take this into account by requiring that combinations of fractions of maximal tolerated doses should be more effective than maximal tolerated doses of the separate drugs. They report that their data suggest that the 6-mercaptopurine-A-methopterin combination is more effective than the separate drugs against L1210. The data, however, are not convincing. In the most extensive experiment reported, the maximal tolerated dose of A-methopterin alone achieved 100 per cent tumor inhibition, and so the combination could show no improvement. When the combination increased survival time the levels employed were at least 50 per cent of the maximal tolerated dose of the separate drugs.</td>
</tr>
</tbody>
</table>

**Motivation:** Drugs were tried on an exploratory basis, alone and together, in an attempt to see if there were any additive, subtractive, or potentiating effects. No specific reason for expecting increased antitumor effect is given. **Criterion:** Fractions of tolerated doses of drugs, in combination, are required to inhibit tumor growth more, and to result in more recoveries from tumor than are obtained with the maximal tolerated levels of the separate drugs alone. Further, the combination should not result in increased toxic mortality. **Summary and Comment:** Data show high inhibition of tumor growth and 45 per cent tumor recovery with a combination of each of the drugs at one-quarter of a tolerated dose. No early deaths occurred with this combination. However, at 2 and 4 times this level of treatment with the drugs in combination, the percentage of early mortality, presumably from toxicity, was 90 and 80 per cent, respectively—this suggests that there is real toxic mortality at the low level, but by chance it did not show up in the current experiment. In contrast, the drugs alone at the higher treatment level, while not evidencing as much antitumor effect, each showed 5 per cent early mortality, and untreated controls 15 per cent. Early deaths with the drugs alone, then, apparently reflect inadequate levels of treatment rather than toxicity. The possibility then exists for duplicating the effectiveness of the combination by raising the level of individual drug treatment.

**Motivation:** Purpose given is that of adding to the knowledge of the potentiating mechanism. **The principle given for combination treatment is that it results in inhibition of several metabolic activities of the tumor. Differences in metabolite concentration between tumor and host may serve as a guide for selecting agents.** **Criterion:** Comparisons are made on the basis of both inhibition of tumor growth and extension of survival time. The addition of flavotin is required to increase the inhibition of tumor growth provided by 8-azaguanine. **Summary and Comment:** Addition of flavotin does result in increased inhibition of tumor growth. This, however, is accompanied by a reduction in average survival time, due primarily to enhanced toxicity. The level of flavotin employed itself produces toxic mortality. Riboflavin-5-phosphate did not reverse the effect of flavotin with respect to either enhanced tumor inhibition or enhanced toxicity.

**REFERENCES**


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The Employment of Combinations of Drugs in the Chemotherapy of Neoplasia: A Review

Abraham Goldin and Nathan Mantel


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