Formal Discussion: Some Enzymatic Considerations in Combination Chemotherapy

R. E. HANDSCHUMACHER[1]

Yale University School of Medicine, New Haven, Connecticut

SUMMARY

A specific example of the effect of two inhibitors acting on a monolinear enzyme sequence in pyrimidine biosynthesis *de novo* has indicated a lack of even an additive effect of this combination. From this, a more general analysis of enzyme kinetics has indicated the difficulty in obtaining potentiation of action at the enzyme level between 2 agents acting in a particular area of metabolism. Some examples are given for those circumstances in which 2 inhibitors do produce a potentiative effect on the growth of microorganisms or tumors for which there are possible biochemical explanations.

The concept that favorable therapeutic actions may be obtained by combinations of drugs is supported by the observed potentiation of drug action on cell growth and reproduction seen with certain pairs of drugs. These findings have often been assumed to reflect either sequential inhibition of a monolinear chain of enzymatic reactions or blockade of each of two independent pathways leading to a common intermediate essential for growth. Recent studies by Dr. R. J. Rubin, Dr. A. Reynard, and myself have given experimental evidence which would question the validity of sequential inhibition of a chain of enzymes as a method of obtaining a potentiation of the action of two inhibitors. Our previous research had established that 5-azaorotic acid and 6-azauridine, after enzymatic conversion to 6-azauridine-5′-phosphate, would block orotidylic acid pyrophosphorylase and orotidylic acid decarboxylase, respectively (Chart 1). These compounds, therefore, were tested in various combinations in anticipation of potentiative inhibition of the growth of Adenocarcinoma 755 and L5178Y lymphoblastic leukemia. In nc case was even an additive effect of these two analogs observed; although tumor inhibition was not extensive, it seemed more important to determine the biochemical effect of these compounds on pyrimidine synthesis *de novo* in cell suspensions and with soluble enzymes. These studies, which have since been published (7), are summarized in Table 1 and Chart 2.

It is apparent from Table 1 that, in isolated leukocytes from a dog, there is not even an additive effect when effective levels of these 2 inhibitors are combined to cause blockade of pyrimidine synthesis *de novo*; subsequently, these studies have been confirmed with suspensions of human leukemic cells. Furthermore, with soluble enzymes, where possible objections of compartmentalization and cellular pools cannot be raised, the result of combining effective levels of each inhibitor was much less than even additive. In Chart 2, the inhibition produced by different levels of 5-azaorotate is shown to be unaffected by 6-azauridine until the azauridine concentration is such that it will produce greater inhibition by itself. These findings have been shown not to be the result of a decreased affinity of 1 inhibitor for the appropriate enzyme in the presence of the other inhibitor or a change in the phosphorylation of 6-azauridine. Webb (9), in a brief consideration of such dual inhibition, also arrived at the same conclusion on the basis of purely theoretical considerations. Thus, as exemplified in Chart 2, the rate of formation of product C is dependent upon the slowest reaction in the sequence, which under control conditions is the conversion of A to B by enzyme 1 (E_1). A system in which the 2nd enzyme (E_2) catalyzed the rate-limiting reaction would not be in a steady state, and the majority of the substrate A would accumulate as intermediate B until the reduced levels of A resulted in a decreased velocity of E_1 to the new rate of E_2 resulting from the enhanced level of intermediate B. Beyond this point, the original conditions of E_1 being rate-limiting in the production of C would be reestablished. Under the first condition shown in Chart 2, introduction of inhibitor X at a level causing 50% inhibition of E_1 will produce a 50% reduction in the rate of product C formation, but further addition of a level of inhibitor Y capable of causing up to 95% inhibition of E_2 will not further suppress the formation of C. *Only when sufficient inhibitor* Y *is added to cause* E_2 *to become the rate-limiting reaction can the effect of this inhibitor be observed.* It must be emphasized that these conclusions are for a closed system from which substrates, intermediates, and inhibitors cannot escape and pertain primarily to steady-state conditions, although their validity can also be established for certain non-steady-state conditions. This analysis is particularly valid for noncompetitive or ti-

[1] Career Professor of Pharmacology, American Cancer Society.

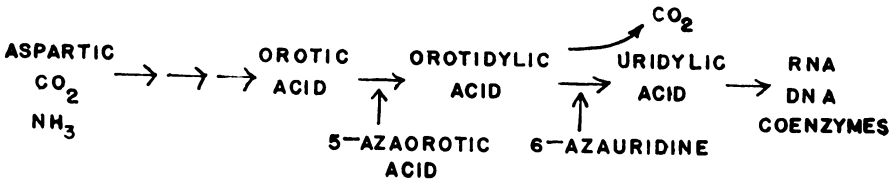


CHART 1.—Sites of inhibition of pyrimidine biosynthesis. RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

TABLE 1

EFFECT OF COMBINATION OF AZAURIDINE AND AZAOROTATE ON THE SEQUENTIAL CONVERSION OF OROTIC ACID-^{14}COOH TO $^{14}CO_2$ AND URIDINE NUCLEOTIDES

System	Inhibitor	Inhibition (%)
Intact leukocytes isolated from dog	6-AzUR[a] (5×10^{-5} M)	49
	5-AzO[a] (5×10^{-5} M)	45
	6-AzUR (5×10^{-5} M) + 5-AzO (5×10^{-5} M)	52
Cell-free supernatant fraction of rat liver	6-AzUR (10^{-6} M)	29
	5-AzO (2.5×10^{-7} M)	43
	6-AzUR (10^{-6} M) + 5-AzO (2.5×10^{-7} M)	47

[a] 6-AzUR, 6-azauridine; 5-AzO, 5-azaorotate.

trating inhibitors. With competitive inhibitors, inhibition of the 2nd reaction will, of course, be partially nullified by the resultant accumulation of the intermediate *B* which would compete with *Y* and further decrease the possibility of even additive inhibition. However, inhibition of the 1st reaction in a sequence in which E_2 is rate-limiting (a non-steady-state condition), so that the rate of accumulation of intermediate *B* is reduced, may enhance temporarily the inhibition of E_2 by *Y* as a competitive inhibitor. This condition is, of course, evanescent since intermediate *B* will soon attain a steady-state level such that inhibition by *Y* will be largely overcome.

Another attractive concept in combination chemotherapy has been that inhibition of each of 2 different pathways that yield a common product essential to growth would result in an inhibition greater than the sum of the inhibition produced by each inhibitor alone. As depicted in Chart 3, it is immediately apparent that the rate of formation of product *C* is the sum of the reactions catalyzed by E_1 and E_2. Therefore, as shown in the example with 50% inhibitory levels of *X* and *Y*, the greatest possible effect of the 2 inhibitors acting simultaneously would be at best equal to the degree of inhibition of the individual enzymes. If the slower reaction rate of E_2 is inhibited to a greater degree (i.e., 90%), the inhibition will be considerably less than the inhibition produced on a single enzyme. These examples are independent of the nature of the inhibitors and the reversibility of E_1 and E_2. Such a consideration illustrates the futility of blocking a minor pathway but also emphasizes the value of having information on the relative importance of 2 convergent metabolic pathways to the growth or function of the tissue under study.

Thus, when potentiation of growth inhibition is observed with 2 inhibitors believed to act either on a monolinear chain or on multiple chains of enzymes responsible for the formation of a compound essential for growth, it

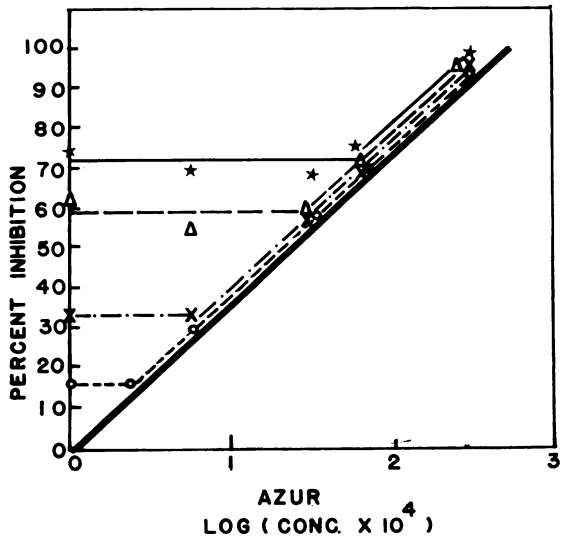
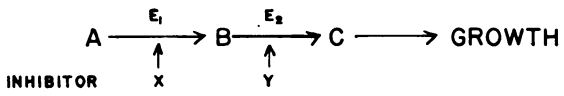


CHART 2.—Kinetics of the sequential blockade of the conversion of orotate to uridylate and CO_2 in a cell-free supernatant fraction of rat liver. The 4 sets of symbols represent the dose-response curves for azauridine (AZUR) in the presence of 4 different levels of azaorotate. The points and lines, where they overlap one another, are moderately displaced for the purpose of clarity. From Rubin *et al.* (7).

is difficult to find an explanation based on the kinetics of isolated enzymes. This, of course, requires that we examine other possible mechanisms by which potentiation has been achieved. Webb has outlined several possibilities (9), and it is now possible to give experimental examples of certain of his cases.

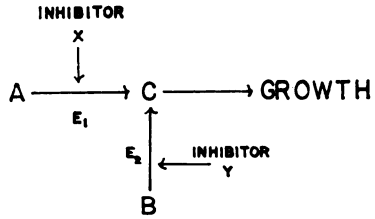
A major restriction on the above considerations is the use of a closed system wherein the enzymes and substrates are contained. In biologic systems, compartmentalization of the enzymes and substrates may result in changed concentration relationships. In addition, substrates and



RATES

	E_1	E_2	C FORMATION
CONTROL	10 MOLES/HR	100 MOLES/HR	10 MOLES/HR
X^1	5	100	5
$X^1 + Y^1$	5	5	5
Y^2	10	2.5	2.5
$X^1 + Y^2$	5	2.5	2.5

CHART 3.—Effects of dual inhibition on a monolinear enzyme sequence.



	E_1	+ E_2	= RATE OF C FORMATION
CONTROL	100 MOLES/HR	10 MOLES/HR	110
$X^1 + Y^1$	50	5	55 (50% INHIBITION)
$X^1 + Y^2$	50	1	51 (54% INHIBITION)

CHART 4.—Effects of multiple pathway inhibition.

intermediates that accumulate when an enzyme is inhibited may either be degraded in the cell or diffuse out of the cell into the circulation. Thus the steady-state condition of a cell is certain to be much more complex than that of the isolated enzyme system. Unfortunately, documented examples of potentiated inhibition with 2 compounds explorable on this basis are not available, although Dr. Nichol has suggested in his presentation ways in which alteration of cell permeability by a 2nd drug might result in potentiation of drug action or at least in greater selectivity of action.

Greater than additive inhibition is most readily seen in those cases in which an inhibitor is degraded by a tissue or animal to an inactive form and this reaction is blocked by the 2nd inhibitor, which may in itself have little if any inhibitory action on growth. Clear examples of this may be seen in the potentiation of the action of 6-mercaptopurine by blockade of xanthine oxidase with 6-hydroxypyrazalopyrimidine (4); the enhanced toxicity of azaguanine (3) in the presence of aminoimidazolecarboxamide, an effective inhibitor of guanase; and the greater activity of 5-fluorouracil and 5-fluorodeoxyuridine in the presence of azathymine or even thymine, which compete with 5-fluorouracil for the uracil catabolic system (1).

Related to this mechanism of enhanced inhibition by 2 inhibitors is the suppression of 1 of 2 convergent metabolic sequences so that the 2nd pathway is enhanced either by derepression or by negative feedback. This sets the stage for more extensive activation of a 2nd inhibitor that creates a metabolic blockade at some distance from the convergent metabolic point. An example may be seen in the blockade of purine synthesis *de novo* with azaserine, which results in greater than normal activation of 6-mercaptopurine or 6-thioguanine to their active forms as nucleotides. Similarly, inhibition of the formation of thymidylic acid either by amethopterin (5) or by 5-fluorodeoxyuridine (6) enhances the phosphorylation of 5-iododeoxyuridine and its subsequent incorporation into nucleic acids.

Another possibility for potentiation of action between 2 inhibitors in whole cell studies, not seen on simple analysis of enzyme kinetics, is inhibition by an inhibitor of protein synthesis of adaptive or derepressed enzyme synthesis induced by an anti-metabolite. Although a clear-cut demonstration of this is not available in mammalian systems, it may be assumed that such inhibition could play a role in the synergism observed by Skipper *et al.* (8) with amethopterin and ethionine or in the enhanced toxicity observed with combinations of 6-azauridine and chloramphenicol (2). Since insensitivity to a given anti-metabolite may reflect a rapid derepression of enzyme synthesis and not a selection of genetically resistant cells, further exploration of this approach would seem warranted with such compounds as actinomycin D, puromycin, ethionine, and chloramphenicol.

Finally, mere potentiation of the biochemical activity of an anti-metabolite by a second inhibitor in no way assures an improved therapeutic effect. Specificity of potentiation for the neoplastic cell is the obvious goal, rather than reduction of the dosage of individual agents to achieve the same toxicity. It will undoubtedly be of great value, however, to achieve a detailed understanding of the biochemical mechanisms by which certain drug combinations do exert synergistic effects on the over-all phenomenon of growth since this could then provide the key for establishing tissue specificity.

REFERENCES

1. Burchenal, J. H., Oettgen, H. F., Reppert, J. A., and Coley, V. Studies on the Synergism of Fluorinated Pyrimidines and Certain Pyrimidine and Purine Derivatives Against Transplanted Mouse Leukemia. Cancer Chemotherapy. Rept., *6:* 1, 1960.
2. Cardoso, S. S., and Jaffe, J. J. Potentiation of the Carcinostatic Action of Azauridine by Chloramphenicol. Biochem. Pharmacol., *8:* 252–55, 1961.
3. Carlo, P. E., and Mandel, H. G. The Effect of 4-Amino-5-imidazolecarboxamide on the Toxicity of 8-Azaguanine. Cancer Res., *14:* 459–62, 1954.
4. Elion, G. B., Callahan, S., Nathan, H., Bieber, S., Rundles, R. W., and Hitchings, G. H. Potentiation by Inhibition of Drug Degradation: 6-Substituted Purines and Xanthine Oxidase. Biochem. Pharmacol., *12:* 85–93, 1963.
5. Hampton, E. G., and Eidinoff, M. L. Administration of 5-Iododeoxyuridine-I^{131} in the Mouse and Rat. Cancer Res., *21:* 345–52, 1962.
6. Kriss, J. P., Tung, L., and Bond, S. The Distribution and Fate of Iododeoxyuridine in the Mouse and Rat. *Ibid.*, *22:* 1257, 1962.
7. Rubin, R. J., Reynard, A., and Handschumacher, R. E. An Analysis of theLack of Drug Synergism during Sequential Blockade of *de novo* Pyrimidine Biosynthesis. *Ibid.*, *24:* 1002–6, 1964.
8. Skipper, H. E., Thomson, J. R., and Bell, M. Attempts at Dual Blocking of Biochemical Events in Cancer Chemotherapy. *Ibid.*, *14:* 503–7, 1954.
9. Webb, J. L. Enzyme and Metabolic Inhibitors, p. 498. New York: Academic Press, Inc., 1963.