Europ. J. Cancer Vol. 17, No. 6, pp. 629–634, 1981
Printed in Great Britain

0014-2964/81/060629-06 $02.00/0

Cytotoxic Effect of 5-Fluorouracil Plus Cyclophosphamide Against Transplantable Leukemias*

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Abstract—*The cytotoxic effect of the combination of cyclophosphamide and 5-fluorouracil against AKR and* L1210 *leukemias was quantitated by a spleen colony assay. We used different sequences, a number of doses of each agent, and different intervals between the agents and noted different degrees of synergistic cell-kill. By proper scheduling of these agents, greater than* 100*-fold increase in cell killing was noted, an effect not demonstrable for normal hematopoietic stem cells. However, the pattern of response for AKR was opposite to that for* L1210 *leukemia; we suggest that this reflects a difference in the metabolism of* 5-FU.

INTRODUCTION

5-FLUOROURACIL (5-FU) and cyclophosphamide (CY) are two of the agents most widely used in the clinical treatment of solid tumors. More and more, these two agents are being studied in combinations which yield increased efficacy [1–3]; however, as with many of the combinations presently in use, their optimal scheduling in terms of dose, sequence and interval between their administration is not known. In this study, we have examined these parameters in two experimental systems, measuring the cytotoxicity to leukemia cells and normal hematopoietic stem cells. We found significant cytotoxic differences depending upon the parameter of dose, sequence and interval; however, because of the difference observed between the two systems studied, we hesitate directly to translate the experimental data to the clinic without pertinent biochemical information as well.

Accepted 19 November 1980.
*This work was supported by N01-CM-4373 and Grant R01CA24960 awarded by the National Cancer Institute, DHEW.
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MATERIALS AND METHODS

Drugs

We purchased 5-FU from Hoffman–LaRoche (Nutley, NJ) in 500 mg vials and CY from Mead Johnson Laboratories (Evansville, IN) in 100 mg vials. The drugs were dissolved and diluted in sterile saline and the specified dose injected in a volume of 0.5 ml via the tail vein.

Mice

AKR mice were obtained from the Jackson Laboratory (Bar Harbor, ME). $CD2F_1$ and DBA/2 mice were obtained through the National Cancer Institute (Bethesda, MD). Mice of either sex were used for the experiments; they were 7–9 weeks old and weighed 19–24 g each.

Leukemia cells

We used the AKR line derived from a spontaneous AKR thymic lymphoma [4]. It was passaged weekly as previously described [5]. The mice received 10^6 leukemia cells via the tail vein 4 days before treatment. The L1210 line, obtained from the NCI in 1971, is maintained by weekly intravenous transplant in male DBA/2 mice. CDF_1 mice received 2

$\times 10^6$ L1210 cells via the tail vein; four days later the drugs were administered.

Assay for leukemia colony-forming units (LCFU)

At specified intervals after the final treatment we killed groups of four leukemic mice by cervical dislocation, removed their femurs and prepared monodispersed suspensions of marrow cells as described previously [5]. We then injected 0.5 ml fractions of this suspension into the tail veins of 8 recipient mice, either AKR or CDF_1 for AKR leukemia and L1210 leukemia, respectively. Eight days later, we removed the spleens of the recipient mice, placed them in Bouin's solution and counted the macroscopic colonies. From this value, we estimated the number of LCFU in the original donor femur [5]. The results were expressed as the fractional survival of LCFU compared to an untreated control group, assayed in the same way, five days after the injection of leukemia cells. To obtain time–survival curves we assayed the femoral marrow for its LCFU content at various time intervals after the administration of the agent and compared these values to those obtained from a control group assayed 4 days after the injection of leukemia cells.

Assay for normal hematopoietic colony-forming units (NCFU)

For this assay, we used 5 normal CDF_1 donors and 15 recipient mice. The recipients received 900 rad total body radiation prior to the injection of femoral marrow suspension. These mice were killed 9 days later and the macroscopic colonies on the spleens were counted [6]. The results were expressed as the fractional survival of NCFU normalized to an untreated control group.

RESULTS

Dose- and time-survival studies

In earlier studies we plotted dose–survival curves for both 5-FU and CY in AKR leukemia [7, 8]. Basing the dosage on these data, we used 0.6 mg per mouse of each agent in the combination experiment, as this dose reduces the survival fraction of LCFU to between 10^{-1} and 10^{-2}. Dose–survival curves for CY and 5-FU in L1210 have also been published [8, 9]. Based upon the same dose consideration as above, we chose 1 mg 5-FU per mouse and 0.5 mg CY per mouse for the combination experiments in L1210 leukemia.

Since the time interval between the administration of the drugs was a variable, a correction factor was necessary to account for the proliferation of leukemia cells during this interval. The extent of correction depends upon the time following the first drug after which regrowth of the tumor population commences. The time-survival curves for 0.5 mg per mouse of CY for both AKR and L1210 leukemia have been published [8]. Recommencement of tumor proliferation was quite rapid following drug exposure, occurring within 2 hr of drug administration. Therefore for all intervals greater than 2 hr, when CY was administered first, a correction of the data was made based on the control growth rate of the tumor population.

The time–survival curves for 0.6 mg per mouse of 5-FU in AKR leukemia and for 1 mg 5-FU in L1210 leukemia have also been published [7, 9]. However, since the interval between between 5-FU and CY in the L1210 combination experiments extended beyond 24 hr, we examined the kinetics of LCFU repopulation with 1 mg of 5-FU. The results are shown in Fig. 1. There was an initial

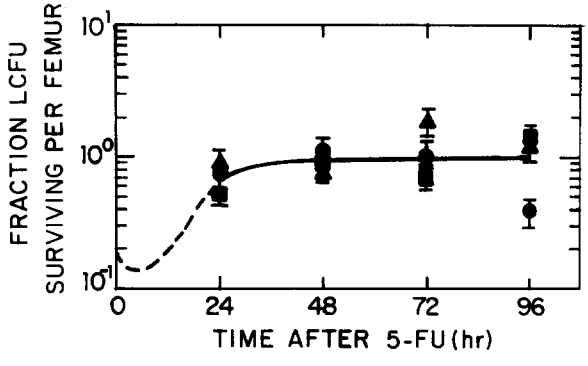

Fig. 1. Survival of LCFU as a function of time following the administration of 1 mg 5-FU *per mouse. Different symbols represent different experiments. Errors are* ±1 S.E. *Dashed line refers to data in* [9].

rapid killing of tumor cells that brought the surviving fraction to a minimum of about 10^{-1} at around 6 hr. LCFU started to proliferate thereafter, but after 24 hr very little, if any repopulation was observed over the subsequent 3 days.

Effect of the combination 5-FU and CY on LCFU

Either drug was given at the doses indicated above, and at time intervals from 5 min to 24 hr later, the other one was administered. Twenty-four hours following the second agent, the femoral marrows were assayed for their LCFU content. The results for AKR leukemia are shown in Fig. 2 with the results for each agent alone represented by solid hexagons. Multiplying the survival for the individual agents alone, we predicted the ad-

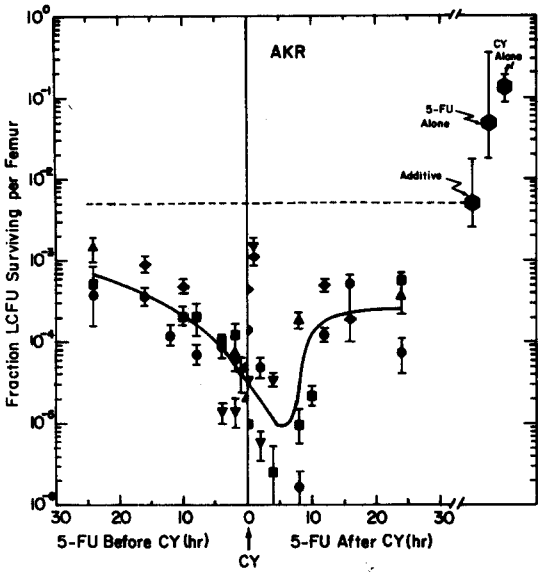

Fig. 2. Survival of AKR LCFU as a function of sequence and interval between the administration of 0.6 mg *per mouse of both* 5-FU *and CY. Hexagons represent the geometric mean of four separate experiments with the limits representing the range of individual values obtained. Different symbols represent different experiments, errors are* ±1 *S.E.*

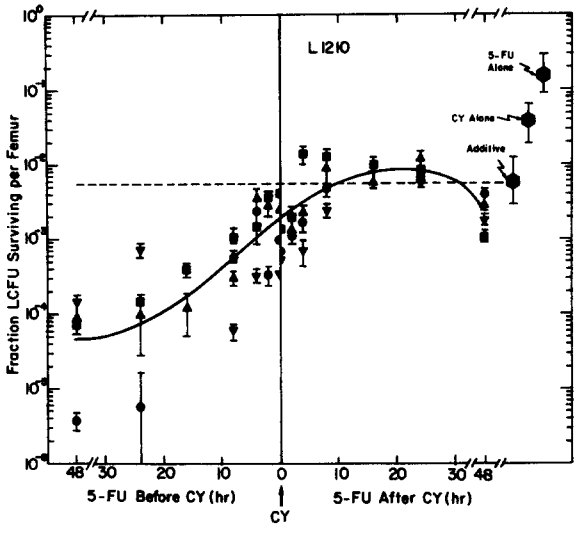

Fig. 3. Survival of L1210 *LCFU as a function of sequence and interval between the administration of* 1 mg *per mouse* 5-FU *and* 0.5 mg *per mouse CY. Symbols and errors as in text (Fig. 2).*

ditive survival for the two drugs to be about 5×10^{-3}. However, when the agents were used in combination, a definite synergism was observed for all intervals and both sequences. For 5-FU administered before CY, the greatest cytotoxicity occurs when they are given close together; as the interval increases, the extent of cytotoxicity decreases and is close to the theoretical additive effect for the 24-hr interval. For 5-FU following CY, cell-killing is maximal at about 10^{-5} between 4 and 8 hr; as the interval increases thereafter, the extent of cytotoxicity decreases but remains greater than additive even at the 24-hr interval.

An identical experiment was then carried out with L1210 leukemia, except that the time interval was extended to 48 hr. The results are shown in Fig. 3. Here also a definitive synergism was observed for most time intervals, but the schedule dependency of this synergism was different from that observed for AKR leukemia. For 5-FU given before CY, the surviving fraction decreased with increasing interval, reaching between 10^{-4} and 10^{-5} at 24 hr. For short intervals, survival was close to the expected additive level of about 5×10^{-3}. Further, when 5-FU followed CY, survival was at about the additive level up to 48 hr.

Effect of the combination 5-FU and CY on NCFU

For these experiments we used the same doses of 5-FU and CY as for the L1210 Leukemia. They were given singly and in combination for the same intervals of time as for the LCFU studies, and the results are shown in Fig. 4. The additive level was about 6 to 7×10^{-1} and the two-drug combination was slightly synergistic for all time intervals studied.

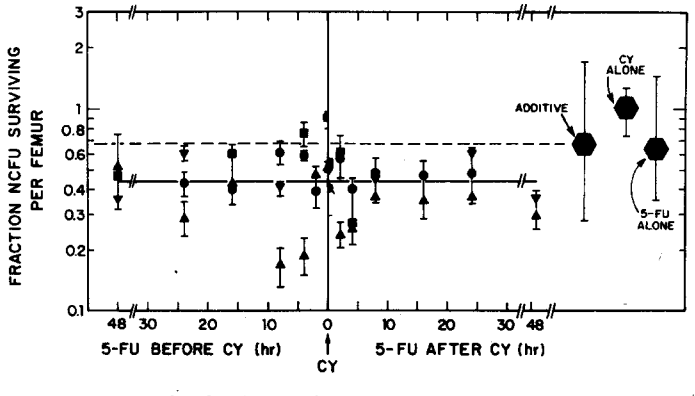

Fig. 4. Survival of NCFU as a function of sequence and interval between the administration of 5-FU *and CY. Doses, symbols and errors as in text (Fig. 2).*

Table 1. Synergy index for the combination of 5-FU and CY in L1210 leukemia*

Dose CY+ (mg/mouse)	Dose 5-FU (mg/mouse) 0	0.25	0.5	1.0	1.5
0	1	2.9×10^{-1}† (1.7×10^{-1} to 4.6×10^{-1})‡	2×10^{-1} (1.5×10^{-1} to 2.5×10^{-1})	9.4×10^{-2} (7.4×10^{-2} to 1.4×10^{-1})	1.9×10^{-2} (9.8×10^{-3} to 4.7×10^{-2})
0.25	1.7×10^{-1} (6.9×10^{-2} to 3×10^{-1})	4.4 0.4 2.4	6.5 11 11	16 3.9 2.1	38 8 24
0.5	3×10^{-2} (1.3×10^{-2} to 9.6×10^{-2})	3 1.8 2.8	4.9 — 5.5	142 61 227	82 156 8.5
0.75	3.7×10^{-3} (1.7×10^{-3} to 5.7×10^{-3})	1.4 1.4 0.6	4.2 17 39	38 38 164	17 9.4 26
1	1×10^{-3} (4.7×10^{-4} to 2.1×10^{-3})	1.7 2.7 1	49 58 13	61 23 27	102 >170 —

*5-FU administered 24 hr before CY.
†Values for each drug alone are the average values from 3–5 separate experiments.
‡Range.

Synergy index

We examined the effect of the dose of each agent on the extent of L1210 cell-killing. We chose a 24-hr interval between drugs with 5-FU given first, since we noted significant synergy for this schedule (Fig. 3). We studied four doses of each agent; the results of five separate experiments are presented in Table 1. The data presented in Table 1 for CY and 5-FU are in agreement with those published previously [8, 9]. It is noted that in neither case is there any evidence of a shoulder region in the dose–survival curves. The synergy index is defined as the ratio of the expected LCFU survival level and the experimental value obtained. The expected level is simply the product of the LCFU survival values for the individual agents alone. While significant variability was noted between experiments, certain generalizations may be made. First, there is little, if any, synergistic effect for the lowest dose of 5-FU combined with any dose of CY. With increasing dose of either 5-FU or CY there is generally an increasing synergistic effect, although the maximum appears to be a factor of 100–200.

DISCUSSION

Two major points of interest emerge from this study. First, the combination of CY and 5-FU demonstrates significant sequence and interval dependency in its cytotoxicity. Synergistic effects of a large magnitude can be obtained in both leukemia models by appropriate scheduling of the agents. Second, the pattern of responses observed for the AKR and L1210 leukemias seems exactly opposite; maximal cytotoxicity for AKR leukemia occurred when 5-FU shortly followed CY, but for L1210 the combination was most effective when 5-FU preceded CY by 24 hr or more.

This latter point may be discouraging since it indicates, on first analysis, that scheduling information cannot be translated from animal to human tumors. However, the different responses probably reflect different ways in which these two cell types metabolize the drugs, with 5-FU most likely responsible for the discrepancy. The sensitivity of various cell lines to 5-FU has been related to rate and extent of nucleotide formation [10], to tumor levels of a pyrimidine phosphoribosyl transferase [11, 12], and to rate of nucleoside transport [13]. 5-FU has a complex anabolic pathway and exerts its cytotoxic effect through at least two main mechanisms: (1) its metabolite 5-FdUMP inhibits DNA synthesis by blocking the enzyme thymidylate synthetase, and (2) it is incorporated into RNA, through conversion to 5-FURTP, though the biological consequences of such incorporation

are not clear [14]. The synergism observed in AKR leukemia when 5-FU is given after CY could be explained by the interference of 5-FU or some of its metabolites with the repair of DNA following the alkylating agent lesion. The dose–survival curve for CY in AKR does indeed exhibit a small shoulder, suggesting accumulation of sublethal injury. The same hypothesis has been invoked to explain the synergism of 5-FU and X-ray in AKR leukemia [7]. Even though there are profound difference between the lesions produced by X-ray and those caused by alkylating agents in the DNA of treated cells [15], it has been demonstrated that alkylation is repaired in mammalian cells [16]. One study suggests that the sublethal damage caused by CY in L1210 ascites tumor could be repaired between 3 and 12 hr after the initial dose.

It is difficult to call upon the same model to explain the synergism observed at different time intervals in both AKR and L1210, but we can assume that the metabolites responsible for the inhibition of the repair reach efficient concentrations at different times after 5-FU exposure in the two cell lines and that the schedule dependencies of the two lines reflect this. For example, 5-FdUMP levels peaked within 1–6 hr in P1534 ascites cells [18] whereas, in the study mentioned above [19], the peak level of 5-FdUMP in solid L1210 was not attained until 24 hr had elapsed.

Another possibility is that 5-FU sensitizes DNA to attack by alkylating agents. 5-FU produces eroded regions in chromosomes of plant cells [20] and chromatid breaks in animals cells [21]. If this visible damage is the ultimate expression of DNA lesions, it would result in a molecular structure more vulnerable to the action of alkylating agents. We have already observed a synergism between L-PAM and 5-FU in L1210 [9] and the schedule dependence was similar to that shown here. Also, a combination of 5-FU and CCNU was recently tested against two experimental solid tumors; 5-FU given 24 hr before CCNU was more effective and less toxic than CCNU followed after 24 hr by 5-FU [22]; this is similar to the data reported here for 5-FU plus CY. The same combination as employed here has been studied in L1210 ascites tumor with therapeutic synergistic noted for the agents given together [23] and more recently, the schedule dependency of 5-FU and CY cytotoxicity was examined in L1210 leukemia, Lewis lung carcinoma and the mouse C22LR osteosarcoma by Mulder *et al.* [24]; the optimum antitumor schedule was found to be that in which the drugs were given simultaneously. The discrepancy between our findings and those of Mulder *et al.* may be due to (1) a difference in the tumor lines; (2) the higher doses of both agents which they used (about twice our dose of 5FU and four times our dose of CY) which may significantly alter the optimal interval; or (3) a difference in the assays used—clonogenic versus increase in lifespan.

All this points to a significant interaction between 5-FU and alkylating agents, but the different schedule-dependent effectiveness of this combination in different tumors cautions us from transferring the results of one experimental model to another before we completely understand the observed phenomenon.

REFERENCES

1. Regelson W. Chemotherapy of gastrointestinal cancer. *Int J Radiat Oncol Biol Phys* 1975; **1:** 109.
2. Bonadonna G, Brusamolino E, Valgussa P. *et al.* Combination chemotherapy as an adjuvant treatment in operable breast cancer. *N Engl J Med* 1976; **294:** 405.
3. Ahman DL, Scanlon PW, Bisel HF. *et al.* Repeated adjuvant chemotherapy with phenylalanine mustard or 5-fluorouracil, cyclophosphamide and prednisone with or without radiation, after mastectomy for breast cancer. *Lancet* 1978; **i:** 893.
4. Bruce WR, Meeker BE. Dissemination and growth of transplanted isologous murine lymphoma cells. *J Natl Cancer Inst* 1964; **32:** 1145.
5. Valeriote FA, Vietti T, Tolen S. Kinetics of the lethal effect of actinomycin D on normal and leukemic cells. *Cancer Res* 1973; **33:** 2658.
6. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse marrow cells. *Radiat Res* 1961; **14:** 213.
7. Vietti T, Eggerding F, Valeriote F. Combined effect of X radiation and 5-fluorouracil on survival of transplanted leukemic cells. *J Natl Cancer Inst* 1971; **147:** 865.

8. Razek A, Vietti T, Valeriote F. Optimum time sequence for the administration of vincristine and cyclophosphamide *in vivo*. *Cancer Res* 1974; **34:** 857.
9. Valeriote F, Vietti T, Coulter D. Synergistic effect of 5-FU and L-PAM on L1210 leukemia. In: Drewinko B, and Humphrey RM, eds. *Growth Kinetics and Biochemical Regulations of Normal and Malignant Cells*. Baltimore: Williams & Wilkins, 1977: 733.
10. Kessel D, Hall TC, Wodinsky I. Nucleotide formation as a determinant of 5-fluorouracil response in mouse leukemias. *Science* 1966; **154:** 911.
11. Reyes P, Hall TC. Synthesis of 5-fluorouridine 5′-phosphate by a pyrimidine phosphoribosyltransferase of mammalian orogin–II. Correlation between the tumor levels of the enzyme and the 5-fluorouracil-promoted increase in survival of tumour-bearing mice. *Biochem Pharmacol* 1960; **18:** 2587.
12. Nahas A, Savlov ED, Hall TC. Phosphoribosyl transferase in colon tumor and normal mucosa as an aid in adjuvant chemotherapy with 5-fluorouracil. *Cancer Chemother Rep* 1974; **58:** 909.
13. Greenberg H, Schumm DE, Webb TE. Uridine kinase activities pyrimidine nucleoside phosphorylation in fluoropyrimidine-sensitive and resistant cell lines of the Novikoff hepatoma. *Biochem J* 1977; **164:** 379.
14. Bujard H, Heidelberger C. Fluorinated pyrimidines XXVII. Attempt to determine transcription errors during the formation of fluorouracil-containing messenger ribonucleic acid. *Biochemistry* 1966; **5:** 3339.
15. Alexander P. Comparison of the mode of action by which some alkylating agents and ionizing radiations kill mammalian cells. *Ann NY Acad Sci* 1969; **163:** 652.
16. Crathorn AR, Roberts JJ. Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. *Nature (Lond)* 1966; **211:** 150.
17. DeWys WD, Kight N. Kinetics of cyclophosphamide damage-sublethal damage repair and cell-cycle-related sensitivity. *J Natl Cancer Inst* 1969; **42:** 155.
18. Myers CE, Young RC, Johns DG, Chabner BA. Assay of 5-fluorodeoxyuridine 5′-monosphosphate and deoxyuridine 5-monophosphate pools following 5-fluorouracil. *Cancer Res* 1974; **34:** 2682.
19. Chadwick M, Rogers WI. The physiological disposition of 5-fluorouracil in mice bearing solid L1210 lymphocytic leukemia. *Cancer Res* 1972; **32:** 1045.
20. Berger CA, Witkus ER. Cytological effects of 5-fluorouracil. *Exp Cell Res* 1962; **27:** 346.
21. Hsu TC, Humphrey RM, Somers CE. Responses of chinese hamster and L cells to 2′deoxy-5-fluoro-uridine and thymidine. *J Natl Cancer Inst* 1964; **32:** 839.
22. Mulder JH, Smink T, van Putten LM. Schedule dependent effectiveness of CCNU and 5-fluorouracil in experimental chemotherapy. *Eur J Cancer* 1977; **13:** 1123.
23. Fernandes DJ, Klubes P. A biochemical and pharmacological study of therapeutic synergism with 5-fluorouracil plus cyclophosphamide in murine L1210 leukemia. *Cancer Res* 1979; **39:** 1396.
24. Mulder JH, Smink T, Ossewaarde T, van Putten LM. Schedule-dependent cytotoxicity of 5-fluorouracil and cyclophosphamide in experimental cancer chemotherapy. *Eur J Cancer* 1980; **16:** 699.