Cancer Chemother. Pharmacol. 4, 221–225 (1980)

Cancer Chemotherapy and Pharmacology

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Selective Uptake and Retention of Anticancer Agents by Sensitive Cells

Donald L. Hill and John A. Montgomery

Southern Research Institute, Kettering-Meyer Laboratory, 2000 Ninth Avenue South, Birmingham, Alabama 35205, USA

Summary. *Considerable evidence has been accumulated to demonstrate that sensitive tumor cells in experimental animals take up and retain at least some effective anticancer drugs to a greater extent than normal tissues, thus providing a greater degree of exposure and accounting for the selective effect of the drugs. In sensitive cells, DNA synthesis is inhibited for prolonged periods, whereas in cells less sensitive the time of inhibition is shorter. In those cases examined where a metabolite, formed intracellularly, is the active form of the agent, the metabolite is produced and is retained to a greater extent than in normal tissues.*

Introduction

For a number of years, those involved in studies of the chemotherapy of cancer have sought to find a biochemical or pharmacological basis for the selective, cytotoxic effect that some chemicals have on some tumor cells. Until recently, progress in this area was almost nonexistent, but a step forward came with delineation of the two types of anticancer agents, those that are effective only against cells that are in a certain stage of the cell cycle and those that are effective against cells regardless of the stage of cycle [24, 25]. It became apparent that cells exposed to a cycle-specific agent would have to remain in the presence of an appreciable concentration of the drug for a time that allowed most of the cells to enter the sensitive phase. Although this information represented a major advance, it still did not adequately elucidate the basis for specificity, for the following reasons:

a) Some normal cell populations divide as rapidly as tumor cell populations [14] and should be equally sensitive to cycle-specific agents.

b) The fact that either of two types of normal stem cells could be the major site of toxicity [10] shows that specificity, not dependent on cell division, exists in regard to such toxic effects.

c) Although some agents kill normal stem cells and cancer cells in roughly equal proportions (and thus have no appreciable antitumor activity), other agents destroy tumor cells effectively with a minimal effect on normal stem cells [3, 27].

d) Some antitumor agents eliminate both resting and dividing tumor cells, in some cases to the same extent [11, 26, 27].

Methotrexate

Methotrexate, an important anticancer agent, is an antifolate, a compound that inhibits dihydrofolate reductase. Inhibition of this enzyme results in a blockade of the synthesis of purine nucleotides and thymidylic acid and subsequent inhibition of synthesis of DNA and cytotoxicity [12]. Normal and tumor tissues contain roughly the same amount of enzyme, and the correlation between enzyme level and responsiveness to methothrexate is poor [1]. In cell-free systems, the dihydrofolate reductases from these sources are inhibited to the same extent by a given concentration of methotrexate [19]. Thus, neither level of enzyme nor differential sensitivity to methotrexate can account for the selective effect on sensitive tumor cells.

For some time, it has been known that both normal and tumor cells possess mechanisms for transporting and concentrating antifolates [9]. It now appears that the selective toxic effect seen for a

Reprint requests should be addressed to: Donald L. Hill

0344-5704/80/0004/0221/$ 01.00

variety of experimental tumor cells is related to differences in this process [6, 19–23].

Following the administration of methotrexate subcutaneously to mice, intraperitonelly transplanted L1210 cells take up the drug, as does the small intestine, the major site of toxicity [19]. Following a dose of 3 mg/kg, however, the leukemia cells maintain levels of methotrexate greater than that required to titrate dihydrofolate reductase and greater than the blood concentration of the agent, for a longer period of time, about 16 h compared with about 3 h for the small intestine. This dose has appreciable antitumor activity when given every other day to these mice. A smaller dose, which gives rise to methotrexate levels in the L1210 cells only slightly greater than the amount required to titrate the reductase, has little or no antitumor effect when administered on the same schedule.

Administered intraperitoneally, methotrexate remained at effective levels in the small intestine for the same period of time but in the L1210 cells for an even longer time, consistent with its greater antileukemic effect when administered by this route [19]. For the small intestine of normal mice, uptake and loss of methotrexate was the same as that for leukemic mice. Increasing the subcutaneous dose of methotrexate beyond the optimal dose reduced the differential retention of the drug by the tumor cells so that, at 400 mg/kg, the difference between these cells and the small intestine was only slight [23].

In accord with the relative retention of methotrexate by the two tissues, inhibition of DNA synthesis, as measured by incorporation of [^{3}H] UdR, was considerably more prolonged in the leukemia cells (greater than 12 h, compared with about 2 h for a dose of 3 mg/kg). Inhibition of DNA synthesis persisted only as long as the intracellular level of drug remained above that required to titrate the enzyme.

The antifolates methasquin and aminopterin, when given as 3 mg/kg doses to mice, remain in the small intestine for a longer time than methotrexate [20]. The longer retention is in keeping with the greater toxicity of these compounds. When the compounds are administered at their LD_{10} doses, however, retention in the small intestine is prolonged and equivalent for all three drugs. At the optimum antileukemic dose, levels in the small intestine remain high for no longer than 3 h [20]. Similar results were observed for 5-chlorodeazaaminopterin [21].

Injection of the four compounds at their optimum antileukemic dose into mice bearing L1210 cells revealed that methotrexate and methasquin were retained at effective levels in the tumor cells for about 24 h; but, in accord with their lesser antitumor activity, aminopterin and 5-chlorodeazaaminopterin were retained for shorter periods [21]. Correspondingly, inhibition of DNA synthesis in the L1210 cells was more prolonged for methotrexate and methasquin than for aminopterin and 5-chlorodeazaaminopterin. At these doses, inhibition of DNA synthesis in the small intestine was brief and similar for all four compounds. 10-Ethylaminopterin, a compound with activity against L1210 cells equivalent to that of methotrexate, was retained in L1210 cells and in small intestine to the same exent as methotrexate [6].

In a comparison of the retention of methotrexate, administered to mice either subcutaneously or intraperitoneally, by five different lines of tumor cells, it was noted that straight lines were obtained on plotting of the half-lives for loss of the methotrexate from the cells with the maximum increase in life-span produced by the drug [21]. The retention and increase in survival time were greatest for L1210 leukemia and less for P288 and P388 leukemias and Ehrlich carcinoma cells. Sarcoma S180 cells, not responsive to methotrexate, retained the drug for the shortest period of time. Accordingly, inhibition of DNA synthesis in the tumor cells was in the order L1210 → P288 → P388 → Ehrlich → S180.

Also in keeping with this sequence is the reciprocal of the influx K_m derived for uptake of methotrexate in in vitro experiments [22]. Further, the steady-state concentrations reached in these cells on exposure to 0.44 μM methotrexate increased in magnitude with the percentage increase in life-span for each of the tumors. For the five lines of tumor cells, efflux values were similar, except for P388, the value for which was somewhat larger. V_{max} values were essentially the same for the different lines.

Although the influx K_m values were similar for methotrexate, aminopterin, and 10-ethylaminopterin uptake into L1210 cells, the influx K_m value for aminopterin uptake by cells isolated from the small intestine of mice was much smaller than those for the other two drugs [6]. The greater affinity for aminopterin by the uptake mechanism in the cells of the small intestine is probably related to the greater toxicity and lower antitumor effect of this agent.

Arabinosylcytosine

1-beta-D-Arabinofuranosylcytosine (AraC) is a cycle-specific agent that requires metabolic activation through phosphorylation to the triphosphate derivative, AraCTP. When [^{3}H] AraC (2.5 mg/kg) was injected subcutaneously into mice bearing L1210

leukemia, a tumor sensitive to AraC, and uptake of the drug was measured in the ascites cells and various normal tissues, it was noted that AraCTP accumulated at high levels, for more than 4 h, in the L1210 cells but was present at comparatively low concentrations in liver, small intestine, and several other tissues [7]. In fact, much of the AraCTP present in liver was apparently due to infiltration of leukemia cells, for this organ from control mice contained even less of this metabolite. For normal tissues, the highest content of AraCTP was observed in the thymus and spleen; but these values were only 30% and 10%, respectively, of that for L1210 cells. There was no appreciable difference in the tumor cell and normal tissue content of Ara-C, AraCMP, or AraCDP. Elevated AraCTP levels in L1210 cells were also observed with doses of 0.025, 0.25, and 2.5 mg/kg [7]. The levels were consistently about 10-fold higher than for liver or small intestine.

Although in 8 h there was no recovery of DNA synthesis, as measured by incorporation of [^{3}H] thymidine, in the L1210 cells of mice given 2.5 mg/kg, there was 50% recovery in the small intestine, the major site for toxicity [7]. For L1210 cells, liver, and small intestine, it was noted that the percentage inhibition of DNA synthesis was related to the tissue content of AraCTP.

Although the authors stated that their results were not consistent with the optimum schedule of doses (every 3 h for eight injections), in that AraCTP levels remained high for more than 4 h, they did point out that relatively high levels of AraCTP (about 10 nmol-eq/kg) were required to inhibit DNA synthesis completely [7]. We note that such a level was not present in L1210 cells after 3 h.

A related study was accomplished with mice bearing L1210 leukemia, P288 leukemia, or Taper hepatoma ascites cells [15]. In this case, the dose was 25 mg [^{3}H] AraC/kg, administered intravenously. Analysis of the tumor cells showed that at 15 min after injection, the AraCTP content of L1210 cells reached a level about 40 times greater than that for the other tumor cells. Although at 1 h the AraCTP content in the P288 and Taper cells had increased to a level about one-third of that in L1210 cells, the AraCTP content of these cells remained well below that for the L1210 cells throughout the 4-h period of observation. Again, there was little difference in the cellular contents of AraC, AraCMP, and AraCDP. These results are in agreement with the responsiveness of the tumor lines to AraC, which is in the order L1210 → P288 → Taper hepatoma. In the small intestine and spleen of these mice, levels of AraCTP were about 20-fold less than in the leukemia cells [15].

An attempt has been made to correlate the retention of AraCTP in human blast cells exposed to AraC in vitro with clinical response to a combination of AraC and anthracyclines [16]. Patients were divided into two categories, depending upon the retention by their cells of AraCTP formed during an exposure to AraC. As a group, those patients whose cells gave high values for retention remained in remission longer than those whose cells showed low retention.

L-Phenylalanine Mustard

L-Phenylalanine mustard (L-PAM) is an alkylating agent, not cycle-specific, widely used as a cancer chemotherapeutic agent. [^{14}C]L-PAM was injected at 10 mg/kg, a therapeutically effective dose, to mice bearing either a line of L1210 leukemia sensitive to L-PAM on one side and a line resistant to L-PAM on the other or a line of P388 leukemia sensitive to L-PAM on one side and a resistant line on the other [2]. After the tumors were harvested, the amount of radioactivity irreversibly bound to macromolecules was measured and the concentration of unchanged L-PAM was determined.

At 0.25, 0.5, 1, and 2 h after injection, macromolecules of the sensitive P388 line contained significantly more irreversibly bound radioactivity than macromolecules of the resistant line; and, except for the earliest time, the same was true for the L1210 sensitive and resistant lines [2]. At all times, the concentration of free L-PAM was higher in the sensitive P388 line than in the resistant line. The sensitive L1210 line contained significantly more L-PAM at 0.25 and 2 h and equivalent amounts at the other times. It is noteworthy that the parent P388 line is more sensitive to L-PAM than the parent L1210 line.

Adriamycin

The Lewis lung carcinoma implanted intramuscularly in mice spreads to the lungs, so that after an appropriate number of days, both the primary tumor and metastases are available for study. Cells in metastases are generally more sensitive to chemotherapeutic agents than those remaining in the primary [17, 18]. When mice with such metastases were injected with adriamycin, a chemotherapeutic agent that exerts its effect through binding to DNA, at any one of three different therapeutic levels, the peak level of the intact drug in the metastases was

more than five times that in the primary [8], and the calculated values for concentration times time ($c \times t$), a measure of exposure, were astoundingly higher for the metastases. Similar results were observed for a single dose level of duanomycin. Higher amounts of hydroxyurea and methylnitrosourea were also observed in the metastases, but the differences were not as dramatic. For methotrexate, the results were equivocal. Cyclophosphamide also accumulated to a greater extent in the metastases; but, even more remarkably, the alkylating metabolites of cyclophosphamide were present in much greater amounts. In our studies of this tumor we found that cyclophosphamide had marked activity, adriamycin had a marginal effect, and hydroxyurea and methotrexate were inactive [13; Schabel FM Jr, unpublished results]. We have not tested methylnitrosourea.

5-Fluorouracil and Fluorodeoxyuridine

5-Fluorouracil is an anticancer agent that cannot adequately be characterized as one that is cycle-specific or one that is not. Intracellularly, it is activated by conversion to the deoxynucleotide, FdUMP, which is a potent inhibitor of the thymidylate synthetase. In one of the earliest, productive studies on selective uptake and retention of anticancer agents, [2-^{14}C]5-FU was injected to mice bearing solid L1210 leukemia, a tumor line moderately sensitive to the drug, at 200 mg/kg, a therapeutic, relatively nontoxic dose [5]. A combination of column and thin-layer chromatography was used to separate the compound and its metabolites.

At 24, 48, and 72 h after treatment the tumor contained more acid-soluble radioactivity than any of several other tissues examined [5], and the active metabolite, FdUMP, was present in the tumor in concentrations considerably higher than in the other tissues. In contrast to results with AraC, for which only the concentration of the active metabolite was elevated in tumor tissue, 5-FU and its other metabolites, with the exception of fluoroureidopropionic acid, were also present in the tumor in concentrations greater than those of normal tissues.

In a similar study, results for 5-fluorodeoxyuridine (FUdR), which gives rise to FdUMP in a single metabolic step of phosphorylation, were similar to those for 5-FU [4]. For mice injected with 760 mg/kg of [2-^{14}C-FUdR], a dose equivalent, on the basis of toxicity, to 200 mg 5-FU/kg, more acid-soluble radioactivity was found in the solid L1210 tumor at 3, 24, and 72 h after injection than in other tissues. At these times, the concentration of FdUMP was 5–7 times higher in the tumor than in the small intestine, a site for toxicity. Following a dose of 380 mg/kg, the concentration of FdUMP in the tumor at 24 h was about 10 times that in the bone marrow, small intestine, kidney, and liver, although only about twice that in the spleen of mice not bearing the tumor. (The spleens and bone marrow of tumor-bearing mice were infiltrated by tumor cells at the time of assay.) As was observed for 5-FU, FUdR, and its other metabolites, with the exception of fluoroureidopropionic acid, were also elevated in the tumor, as compared to other tissues [4].

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Received March 12/Accepted April 23, 1980