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Kinetic Analysis of 5-Fluorouracil Action against Various Cancer Cells

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Based on our recent kinetic analysis, which made it possible to distinguish between the cell-killing actions of cell cycle phase-specific and non-specific agents, we attempted to elucidate the actions of 5-fluorouracil (FUra) on three different cancer cell lines. By colony-forming assay, the concentrations of fluorouridine (FUrd), fluorodeoxyuridine (FdUrd) or FUra giving 90% cell kill (IC_{90}) at various exposure times (t_{expe}) were obtained. With P388 cells, the curve of t_{expe}-IC_{90} for FUrd on a log-log scale was linear with a slope of -1, which is typical for cell cycle phase-nonspecific agents. In contrast, the curve for FdUrd showed a much steeper slope than -1, which is characteristic for cell cycle phase-specific agents. We found that the curve for FUra was exactly the same as that for FUrd, indicating that the mode of FUra action on P388 leukemia is analogous to that of FUrd. Similar results were observed with human colon and renal cancer cell lines, HT-29 and KU-2, although when the cells were exposed to relatively low concentrations of FUra for a long time, a cell cycle phase-specific action became evident.

Key words: 5-Fluorouracil — 5-Fluorouracil nucleosides — Cell-killing action — Kinetic analysis

Very recently, our theoretical and experimental studies[1)] based on kinetic analysis revealed that the cell-killing action of cell cycle phase-nonspecific agents depends on the concentration-time product, or AUC.[3] Accordingly, the curves for exposure times and the corresponding IC_{90}'s of these drugs on a log scale were shown to be linear with a slope of -1 when such agents were stable in the culture medium. In the case of unstable drugs, they develop an asymptotic curve having a linear portion with a slope of -1. In contrast, the cell-killing action of cell cycle phase-specific agents is AUC-independent and shows a strong tendency toward time-dependence, presenting curves with much steeper slope in the log-log graph for the exposure times and IC_{90}'s.[2)] These studies indicated that such kinetic analysis is very useful to determine the presence or absence of cell cycle phase-dependence of cell killing action for a given drug.

In the present study, we attempted to make this type of kinetic analysis on the actions of FdUrd, FUrd and FUra. It is reasonably expected that the major action of FdUrd would proceed through FdUMP, thereby being highly specific to the S-phase. On the other hand, we assume that FUrd would act through FUTP preferentially, suggesting its independence of a specific cell cycle phase, because its fraudulent incorporation into RNA is irreversible and probably proceeds in any phase of the cell cycle. These assumptions suggest that FdUrd would present a kinetic pattern like that of a cell cycle phase-specific agent, and FUrd, one like that of a cell cycle phase-nonspecific agent. If this is the case, it would be of much interest to know what type of kinetic behavior the cell-killing action of FUra would present. Thus, we conducted a kinetic analysis of FdUrd, FUrd, and FUra against murine leukemia P388, human colon cancer HT-29, and human renal cell cancer KU-2 cells.

MATERIALS AND METHODS

Chemicals FUra was kindly supplied by Kyowa Hakko Kogyo Co., Ltd., Tokyo. FUrd was a gift from Dr. Ohashi, Research Institute for Polymers and Textiles, Tsukuba, who chemically synthesized it. FdUrd was purchased from Sigma Chemical Co., St. Louis, MO.

Tumor cells P388 leukemia was supplied by the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD. In this study, its culture line derived from the original cell population transferred *in vivo* was used. HT-29, a human colorectal carcinoma cell line, was supplied by American Type Culture Collection, Rockville, MD. KU-2, a human renal carcinoma cell line, was kindly provided by Dr. Tazaki, Department of Urology, School of Medicine, Keio University, Tokyo.

Colony-forming inhibition assay P388 cells were cultured on soft agarose. In detail, 0.25 ml of 0.3% agarose

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[3] The abbreviations used are: FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylate; FUTP, 5-fluorouridine-5′-triphosphate; dThd, thymidine; Urd, uridine; dTMP synthase, thymidylate synthase; IC_{90}, concentration necessary for 90% cell kill; t_{expe}, exposure time; AUC, the area under the drug concentration-time curve.

(Sea Plaque; FMC Corporation, Rockland, ME) prepared by mixing 1 vol of melted 3% agarose with 9 vol of RPMI1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum (M.A. Bioproducts) and 100 μg/ml kanamycin (Meiji Seika Kaisha Ltd., Tokyo) was added to each well of a 24-well plate (Corning Laboratory Sciences Co., NY) as an underlayer, and the plates were then refrigerated for 1 h at 4°C. This layer was overlaid with 0.25 ml of a mixture consisting of cell suspension (2,000 cells already treated with the drug for a certain period of time at 37°C) and 3% agarose at the volume ratio of 9:1, and the plates were again refrigerated for 1 h at 4°C. The plates were then transferred to a CO_2 incubator and the cells were cultured for 6 days at 37°C, after which 40 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide solution (5 mg/ml of distilled water) was added to each well. Colonies were stained after further incubation for 4 h at 37°C.

HT-29 and KU-2 cells were cultured on plastic dishes without agar. Cells harvested by trypsinization were seeded at appropriate cell densities on 60-mm dishes (Corning Laboratory Sciences Co.) so as to give a total culture medium volume of 3.15 ml. RPMI 1640 was used for HT-29 cells, and MEM for KU-2, as the culture medium. Both media were supplemented with 10% fetal calf serum, and MEM only was also supplemented with 2 m*M* L-glutamine. On the day after seeding, 0.35 ml of drug solution was added to each dish, and the cultures were then incubated further for various lengths of time in the presence of the drug. At the end of the incubation, the plates were washed twice with 3 ml of phosphate-buffered saline, and then 3 ml of culture medium was added to each. On the 7th and 8–9th day for HT-29 and KU-2 cells, respectively, after seeding, all dishes were washed once with phosphate-buffered saline, fixed with 10% formalin, and stained with 0.05% crystal violet.

Colonies were enumerated by means of a Colony Analyzer CA-7 (Oriental Instruments Ltd., Tokyo). The surviving fraction was calculated by dividing the colony number of cells exposed to the drug by that of the control. The IC_{90} value of each drug was determined from the dose-response curve for each exposure period.

RESULTS

P388 cells in suspension were exposed to various concentrations of FdUrd, FUrd, or FUra for certain periods of time, and then cultured on soft agarose for the colony assay. As a result, dose-response (concentration-survival fraction) curves were made, and IC_{90} values for each exposure time were obtained. These paired data were plotted on a log-log scale as shown in Fig. 1. When cells were exposed to FdUrd, IC_{90} values sharply decreased with increasing exposure time, theraby presenting a linear curve with a much steeper slope than -1 (Fig. 1-A), as we had expected. This is typical for a cell cycle phase-specific drug.[2] In contrast, the curve for FUrd was linear with a slope of -1 (Fig. 1-B), which is typical for cell cycle phase-nonspecific drugs[1,2] and implies a concentration × time-dependent cell-killing action of this drug against P388 leukemia. After confirming these results, we obtained the curve of t_{exp}-IC_{90} for FUra, which is shown in Fig. 1-C. Although IC_{90} values of FUra for the respective exposure times were much greater than those of FUrd, the observed curve had exactly the same

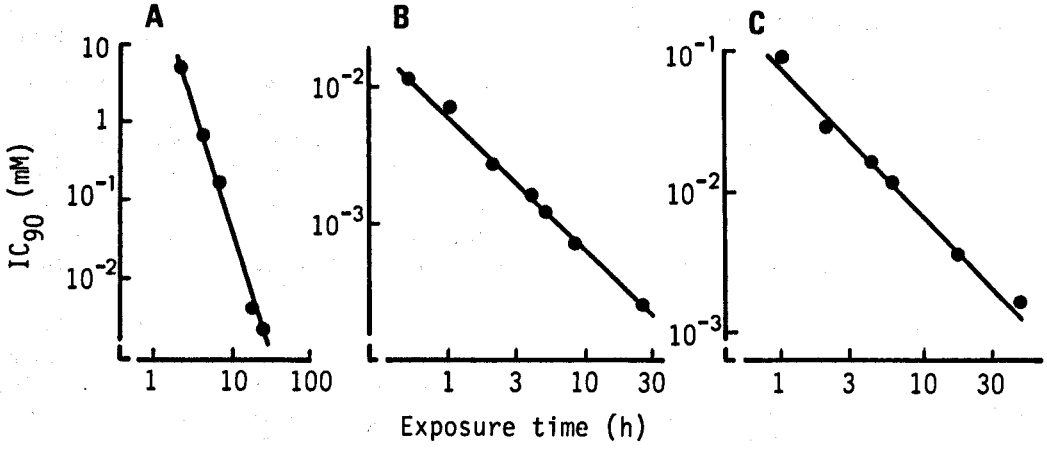

Fig. 1. Log-log relationship between IC_{90} value and exposure time for FdUrd, FUrd, and FUra against P388 leukemia cells. IC_{90} values obtained from the concentration-survival curves for FdUrd (A), FUrd (B), and FUra (C) were plotted against exposure times, each on a log scale. Each value is the mean of 3 or more determinations with a coefficient of variation of less than 20%.

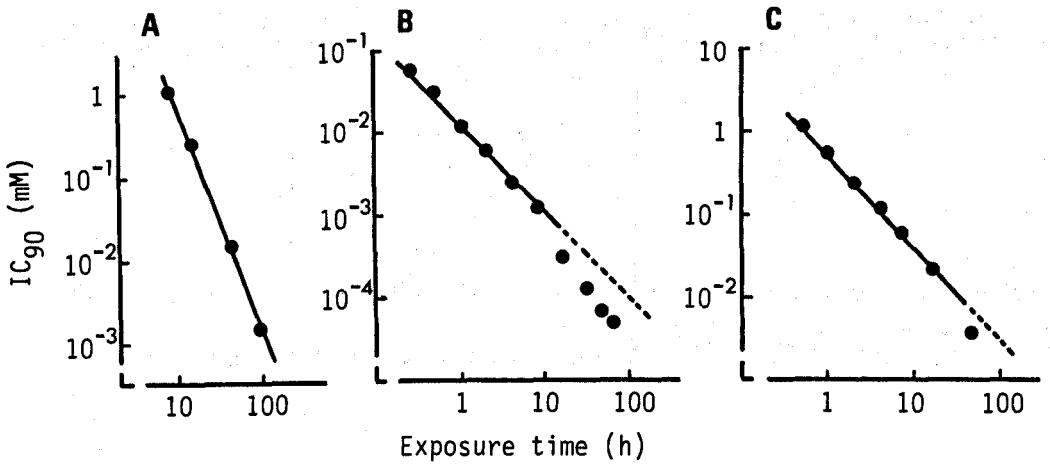

Fig. 2. Log-log relationship between IC_{90} value and exposure time for FdUrd, FUrd, and FUra against HT-29 human colon cancer. For details, see the legend to Fig. 1.

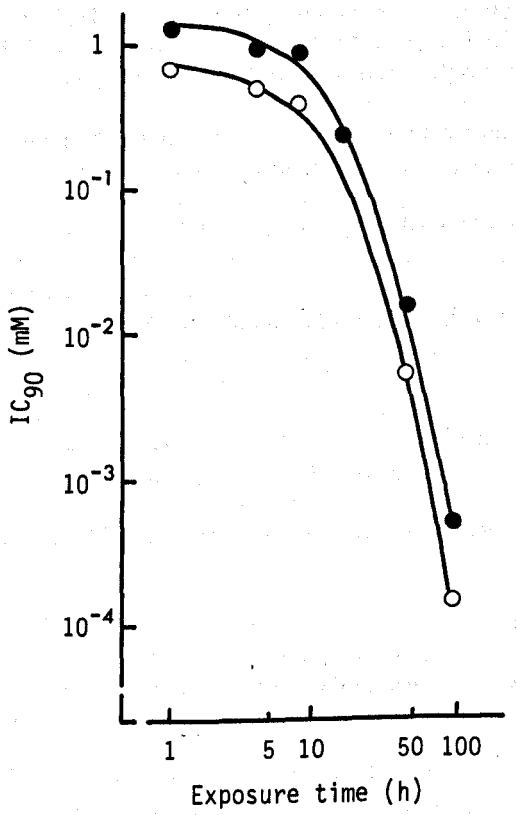

Fig. 3. Log-log relationship between IC_{90} value and exposure time for FdUrd against HT-29 human colon cancer grown in the medium containing dialyzed fetal calf serum. For details, see the legend to Fig. 1. In this experiment, dialyzed fetal calf serum was used and the final concentration of dThd in the medium was adjusted so as to be 0.2 μM. ●, use of undialyzed fetal calf serum; ○, use of the dialyzed serum.

pattern as that for FUrd. This result suggests that the mechanism of the cell-killing action of FUra is identical with that of FUrd, at least under the present experimental conditions.

In the case of HT-29 cells, FdUrd also showed a linear plot with a steeper slope than -1 (Fig. 2-A), similar to the result with P388 cells. However, a significantly longer exposure time was needed to obtain the same IC_{90} values as found with P388 cells. The curve for FUrd was linear with a slope of -1, but it appeared steeper with exposure times longer than about 10 h (Fig. 2-B). This suggests that when HT-29 cells are exposed over a long period to very low concentrations of FUrd, even FUrd exhibits a cell cycle phase-specific action. With HT-29 cells as well, FUra showed the same curve for log t_{exps}-log IC_{90} as FUrd (Fig. 2-C), indicating that FUra acts on HT-29 cells in the same mode as FUrd. Since dThd concentration in fetal calf serum is known to be much higher than that in human serum, log-log relationships between t_{exps} and IC_{90} for FdUrd and FUra were observed by use of dialyzed calf serum. The final concentration of dThd in the medium was adjusted so as to be 0.2 μM. As shown in Fig. 3, IC_{90}'s of FdUrd in the dialyzed serum are somewhat lower than those in the non-dialyzed serum, but the slopes of the two plots are not different. In the case of FUra, both IC_{90} and the slope of the curve remained unchanged when the dialyzed fetal calf serum was used (data not shown). These results indicate that their action on HT-29 cells were the same at both dThd concentrations used.

With KU-2 cells, the curve for FdUrd was biphasic, being linear with a slope of -1 at relatively high drug concentration and short exposure time and having a

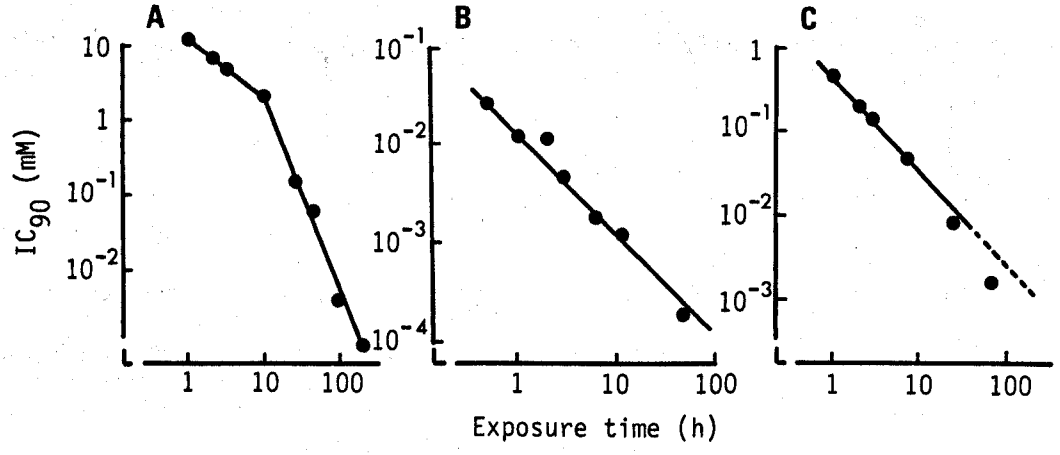

Fig. 4. Log-log relationship between IC_{90} value and exposure time for FdUrd, FUrd, and FUra against KU-2 human renal cancer. For details, see the legend to Fig. 1.

markedly steeper slope at lower drug concentration and longer exposure time (Fig. 4-A). This pattern suggests that the cell-killing action of FdUrd against KU-2 cells changes from a cell cycle phase-nonspecific mode to a phase-specific mode with decreasing FdUrd concentration and increasing exposure time. On the other hand, the curve for FUrd was linear with a slope of -1 (Fig. 4-B). As compared with the curves for FdUrd and FUrd, that for FUra was basically the same as that for FUrd, although it tended to show a steeper slope at lower drug concentraion and longer exposure time (Fig. 4-C). As a whole, the action of FUra on KU-2 cells was very similar to that on HT-29 cells.

DISCUSSION

To elucidate the actual mode of cell-killing action of FUra, various biochemical approaches have been made so far. Based on these studies, RNA-directed effects through the incorporation of fluorouracil into RNA have been reported in Novikoff hepatoma,[3,4] HeLa,[5] KB,[5] Hep-2,[5] Lovo,[6] MCF-7,[7] $CD8F_1$ mammary carcinoma,[8] colon 26,[8] HT-29,[9,10] Friend erythroleukemia,[11] HuTu80,[12] dTMP synthase-deficient FM3A,[13] L1210,[14] and S-180.[15,16] On the other hand, DNA-directed effects through the inhibition of dTMP synthase have also been implicated in L1210,[17] human colon adenocarcinoma xenografts,[18-21] S-180,[22-24] HT-29,[12] colon 38,[25] and clinical specimens of colon and breast cancer.[26]

However, it is very difficult to assess exactly the mode of cell-killing action of FUra based on biochemical changes observed, because we can hardly determine how much such changes, for example, the inhibition of dTMP synthase and/or its fraudulent incorporation into RNA, contribute to the actual cell-killing effects of FUra in individual cancers. In this regard, Keyomarsi and Moran have recently reported that L1210 cells can grow at the normal rate even when dTMP synthase activity is significantly suppressed.[27] Such difficulties in determining the mode of FUra action biochemically might lead to the conflicting conclusions reported on L1210, HT-29 and S-180, as already shown. From this point of view, in the present study, we attempted to assess FUra action based on kinetic analysis of its cell-killing action itself instead of indirect biochemical analysis.

As a result, we successfully distinguished between the kinetic behaviors of FdUrd and FUrd actions against P388 leukemia, and identified cell cycle phase-specific and -nonspecific modes of action for FdUrd and FUrd, respectively (Fig. 1-A, B). These results showed that this type of analysis is available for elucidation of the cytotoxic actions of fluoropyrimidines. The subsequent kinetic analysis of FUra revealed that this drug acts exactly as FUrd does on P388 leukemia (Fig. 1-C). According to our assumption already noted, this suggests that such cell cycle phase-nonspecific action of FUra against P388 leukemia is predominantly RNA-directed, and dependent on AUC. In this connection, Byuyan *et al.*,[28] using synchronous Chinese hamster fibroblast DON cells, found that FdUrd showed S-phase specific action but FUra was equally cytotoxic to cells in all phases of the cell cycle. Ardalan *et al.*[29] investigated enzyme activities involved in FUra anabolism in P388 leukemia, and found that Urd phosphorylase activity is significantly higher than the activities of dThd phosphorylase and pyrimidine phosphoribosyl transferase and, in addition, Urd kinase activity is also remarkably

greater than that of dThd kinase. The enzyme activities observed do not directly indicate the actual metabolic pathway of FUra since they were measured with artificial concentrations of cofactors. However, these biochemical data, at least, are not contradictory to the result of our kinetic analysis. On the other hand, it has been generally accepted that FUra, different from 1-β-D-arabinofuranosylcytosine, does not show a definite schedule-dependence in the treatment of P388-bearing mice. This also agrees with the AUC-dependent action of FUra against P388 leukemia.

With a human colon cancer, HT-29, similar results were obtained (Fig. 2). In contrast to P388 leukemia, in which FUra action was predominantly cell cycle phase-nonspecific independently of its concentration and exposure time as far as examined, a cell cycle phase-specific action became evident when HT-29 cells were exposed to relatively low concentrations of either FUrd or FUra for a long time. The longer the exposure time is, the greater the cell cycle phase-specific action of FUra is expected to be at very low cencentrations. Glazer and co-workers[9, 10] suggested RNA-directed effects of FUra on HT-29 cells based on their observations of significant correspondences between the amount of drug incorporated into nuclear RNA and cell lethality and the inhibition of rRNA processing by FUra, respectively. On the contrary, Washtien[12] placed emphasis on a DNA-directed effect of FUra on HT-29 cells mainly because the growth-inhibitory action of FUra was significantly reversed by dThd in 72-h to 96-h drug exposure experiments. In fact, growth inhibition of HT-29 cells by relatively low concentrations of FUra was clearly reversed. However, the higher the FUra concentration became, the less the degree of reversal was. In spite of the fact that the inhibition of dTMP synthase was already maximum at the 50% growth-inhibitory concentration (0.3 μM) of FUra, growth inhibition proceeded further at higher concentrations and was accompanied with an increase in FUra incorporation into RNA. These results might suggest that mild growth inhibition by relatively low concentrations of FUra is caused by the suppressed dTMP synthase activity, but the RNA-directed effect makes a major contribution to severe growth inhibition even in this long-exposure experiment. Therefore, the result of our kinetic analysis is in good accordance with all the reported results.

FUra action against the human renal cancer line KU-2 was quite similar to that against HT-29 cells; when cells were exposed to it for a long time, cell cycle phase-specific action, that is DNA-directed, was partially observed (Fig. 4). It was unexpected that FdUrd would exhibit a cell cycle phase-nonspecific action even with a short-term exposure to relatively high concentrations of it (Fig. 4-A). These data suggest that FdUrd is efficiently converted kinto FUTP probably through FUra and incorporated into RNA in KU-2 cells. The incorporation into RNA of radioactive FdUrd and FUra to the same extent supported this assumption (data not shown).

In conclusion, FdUrd, FUra and FUrd presented characteristic patterns in the kinetic analysis of their cell-killing actions. In particular, FUra showed differing patterns depending on the cancer cell lines, exposure times and drug concentraions used. It seems likely that the mode of *in vivo* antitumor action of FUra depends on the biochemical characteristics of the cancer treated and its pharmacokinetics. It seems that FUra exerts both RNA- and DNA-directed actions in most cases, although their relative potency would be different in each case. Such dual actions could be partially regulated by not only varying the treatment schedule or method but also by using biochemical modulators such as leucovorin. Numerous studies on biochemical aspects of FUra actions have been reported so far. However, the present kinetic analysis on the cell-killing action itself provides a unique insight into the mode of antitumor action of FUra. We are currently studying the biochemical modes of FUra action on these cell lines in connection with our kinetic findings.

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