Cancer Chemother Pharmacol (2000) 45: 38–42

ORIGINAL ARTICLE

Tu T. Aung · Mary A. Davis · William D. Ensminger
Theodore S. Lawrence

Interaction between gemcitabine and mitomycin-C in vitro

Received: 19 November 1998 / Accepted: 21 June 1999

Abstract Both gemcitabine (2′,2′-difluorodeoxycytidine; dFdCyd) and mitomycin-C (MMC) are active against several solid malignancies. dFdCyd is an attractive agent for use in combination with drugs which damage DNA and with radiation therapy because of its ability to inhibit DNA replication and repair as well as its radiosensitizing effect. We hypothesized that the repair of MMC adducts in DNA might be inhibited by dFdCyd leading to a synergistic effect. To test this possibility, we studied the effect of combining dFdCyd and MMC in HT29 human colon carcinoma cells in vitro. The cells were exposed to a variety of drug concentration ratios and schedules, then assessed for clonogenic survival. D_{50} values (drug concentration at which clonogenicity is inhibited by 50%) were calculated, and the interactive effects of the two drugs were evaluated using median effect analysis. In this approach, if the calculated combination index (CI) is <1, 1, or >1, it indicates synergism, additivity, or antagonism, respectively (Chou and Talalay 1984). We found that marked synergy (CI of 0.5–0.7) was produced by concurrent exposure to mitomycin and gemcitabine. In contrast, sequential treatment led only to additivity. These findings suggest that, when combined in an appropriate schedule, the chemosensitizing effect of gemcitabine may be beneficial in the treatment of malignancies which are sensitive to MMC.

Key words Gemcitabine (dFdCyd) · Mitomycin-C (MMC) · Colorectal cancer

T.T. Aung · W.D. Ensminger
Division of Hematology/Oncology,
Department of Internal Medicine,
University of Michigan,
Ann Arbor, Mich., USA

M.A. Davis · T.S. Lawrence (✉)
Department of Radiation Oncology,
B2C490 University of Michigan Medical Center,
1500 E. Medical Center Drive,
Ann Arbor, MI 48109, USA
e-mail: tsl@umich.edu
Tel.: +1-734-647-9955; Fax: +1-734-763-7371

Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine; dFdCyd), an antimetabolite, is a fluorinated deoxycytidine analog with preclinical activity in experimental tumor models as well as an impressive clinical activity in solid tumors when used singly or in combination [2, 4, 7, 15, 17, 21, 24]. Mitomycin-C (MMC), a bifunctional alkylating agent, has shown therapeutic activity against a variety of cancers especially in combination with other agents [8, 34]. To date, little is known about the potential effect of the combination dFdCyd and MMC. Only one in vitro study has been reported using this combination of drugs on a Lewis lung (LL) non-small-cell lung cancer cell line [22].

A crucial step in dFdCyd cytotoxicity is its phosphorylation by deoxycytidine kinase [14]. The resulting phosphorylated metabolites exhibit multiple cellular effects: (a) they prevent DNA synthesis by inhibiting DNA polymerases and by competing with deoxycytidine triphosphate [16, 25], (b) they deplete nucleotide pools by inhibiting ribonucleotide reductase [3, 29], and (c) they decrease the accuracy of DNA replication and repair by incorporation into DNA [16, 27]. In addition to its cytotoxic activity, dFdCyd is a potent radiation sensitizer for a variety of human tumor cell lines [28]. Thus, dFdCyd which is cell cycle specific, is an attractive agent for use in combination with DNA-damaging drugs and radiation therapy. On the other hand, MMC, a bioreductive agent which is not cell cycle specific in its actions, is unique among the alkylating agents in that it can be metabolized to an active species which crosslinks complementary DNA strands, thereby inhibiting DNA synthesis [9]. After bioreduction and chemical reduction of MMC has occurred, guanine is the preferred site of alkylation of DNA [33], but this crosslinking is slowly repairable [20].

Since both dFdCyd and MMC have been reported to have enhanced activity in combination with other DNA-damaging agents [6, 11, 13, 26, 32], we postulated that dFdCyd and MMC together may have complementary activity, leading to a selective synergism against the

targeted tumor cells. To test this hypothesis, we studied the effect of combining dFdCyd and MMC in HT29 human colon carcinoma cells in vitro. Our goal was to establish whether the combination of these two drugs has a synergistic effect and if any schedule dependency exists. In assessing schedule, we wished to test both the dependence on the order of drug treatments and the effect of delaying the determination of clonogenic survival for 24 h after dFdCyd treatment. This latter part of the design derived from our finding that dFdCyd cytotoxicity and radiosensitization were increased under these conditions [19]. We found that concurrent exposure to dFdCyd and MMC produced the most synergistic effect in vitro. This finding supports the development of clinical trials using concurrent dFdCyd and MMC for the treatment of solid malignancies.

Materials and methods

Cell culture/drugs

Human colon adenocarcinoma cells (HT29) from the American Type Culture Collection (Manassas, Va.) were cultured in RPMI-1640 medium supplemented with glutamine (10 m*M*), penicillin (100 IU/ml), and 10% bovine calf serum. Cells were maintained at 37 °C in a humidified atmosphere of 7% CO_2 and 93% air as a monolayer under conditions allowing for exponential growth. Under these conditions the plating efficiency of HT29 cells was 70–90%, and the doubling time was approximately 21 h. dFdCyd (Eli Lilly Research Laboratories, Indianapolis, Ind.) was dissolved in phosphate-buffered saline (PBS), and all exposures were for 2 h at 37 °C. MMC (Bristol Laboratories, Princeton, N.J.) for clinical use (40 mg/80 ml) was dissolved in PBS, and all incubations were carried out for 1 h at 37 °C. Incubations were carried out with the drugs diluted from frozen stocks which were made fresh every 3 months. In experiments where the dishes were to be processed 24 h after the end of the drug exposure, the drug-containing medium was removed, the dishes washed with PBS, and fresh medium added.

Cell survival assay

Cell survival was assessed following exposure to dFdCyd and MMC using a standard clonogenic assay as described previously [18]. Briefly, at the time of processing, the medium was removed, the dishes were washed with PBS, and the cells were removed from the plate using trypsin. After the cells had been removed from the dishes, they were counted and diluted into dishes at numbers that were anticipated to produce between 20 and 200 colonies per plate. Appropriate controls of each drug alone were performed in all cases. All experiments were repeated at least three times, with the results expressed as means ± standard error of at least three experiments. In order to apply the median effect analysis (see below), data were fitted using the equation:

$$\text{Surviving fraction} = C_{50}^{M}/(C_{50}^{M} + C^{M})$$

where C is the concentration of drug, C_{50} is the concentration that reduces the surviving fraction to 50%, and M is the slope of the sigmoid curve. The fit was performed using a logarithmic transformation to linearize the equation, as described by Chou and Talalay [5]. We also calculated the linear area under the cell survival curve in a calculation analogous to that used to estimate radiation sensitivity [10]. A smaller area suggests greater sensitivity to the drug condition.

Analysis of cell survival assay

In a first set of experiments, a low, noncytotoxic fixed concentration of dFdCyd and variable doses of MMC were used. The sensitivity of the cells to drug was expressed by calculating the area under the cell survival curve, so that a smaller area indicates greater sensitivity. Potential enhancement of MMC cytotoxicity by dFdCyd was expressed by calculating the ratio of the area under the curve under control conditions divided by the area under dFdCyd-exposed conditions, so that a number greater than 1 would indicate increased sensitivity. In a second set of experiments, combinations of dFdCyd and MMC in various cytotoxic ranges of concentration were analyzed according to the median effect principle as described by Chou and Talalay [5]. The interaction of the two chemotherapeutic agents was then quantified by calculating the combination index (CI). The CI can be calculated by assuming a mutually nonexclusive interaction.

Within the methodology of the median effect calculation, there is a need to determine whether an interaction is "mutually exclusive" or "non-mutually exclusive". Our calculation of the CI as non-mutually exclusive presupposes no assumptions about the mechanism of killing by either drug. This approach adds a term to the CI (D_1D_2/Dx_1Dx_2) compared to the mutually exclusive assumption of interaction and of isobologram analysis [5]. Since higher CIs are interpreted as indicating less enhancement (or even antagonism when the index is greater than 1), we feel that we have made a conservative estimate of the enhancement by using the non-mutually exclusive assumption [5] by the equation:

$$CI = (D_1/Dx_1) + (D_2/Dx_2) + (D_1D_2/Dx_1Dx_2)$$

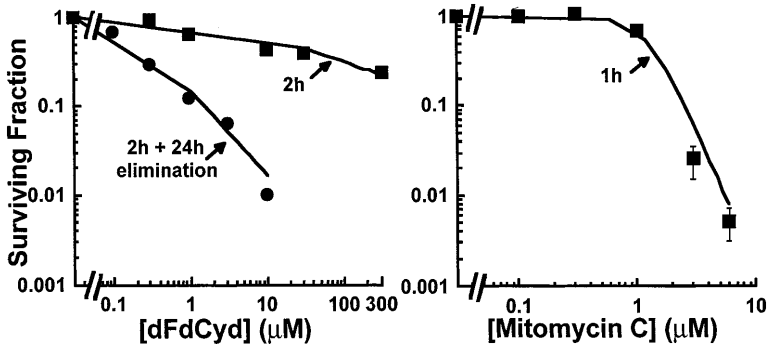

Fig. 1A,B Effect of dFdCyd and MMC on the clonogenic survival of HT29 colon cancer cells. **A** Cells were exposed to dFdCyd for 2 h, the drug was washed from the medium, and the cells were then processed for clonogenic survival immediately (□) or 24 h later (■) as described in Material and methods. **B** Cells were exposed to MMC for 1 h and then processed for clonogenic survival as described in Materials and methods. The values are the mean ± standard error of at least three experiments

where Dx_1 and Dx_2 are the concentrations of drugs 1 and 2, when used alone which produce a surviving fraction of x, and D_1 and D_2 are the individual concentrations of drugs 1 and 2 in the fixed ratio which produces a surviving fraction of x. In this combination model, an antagonistic relationship between the agents is implied when the CI value is greater than 1, while a CI value of 1 indicates an additive effect. A CI value less than 1 denotes synergism.

Statistical analysis

Data are expressed as the means ± standard error of at least three experiments. Means were compared using Student's *t*-test.

Results

Our experimental design was derived from our experiments using dFdCyd with radiation. In these studies we found that dFdCyd could radiosensitize under noncytotoxic conditions. Therefore, we began by determining the cytotoxicity of dFdCyd and of MMC against HT29 cells. For dFdCyd, cells were treated over a 2-h period. The drug was then washed from the medium, and the cells were assessed for clonogenic survival immediately or 24 h later. This time period was chosen because the effects of dFdCyd on dNTP pool depletion occur over the first 30 min and have plateaued by 2 h [14]. For MMC, clonogenicity was determined immediately after a 1-h drug exposure (Fig. 1). We found that the D_{50} (drug concentration at which clonogenicity is inhibited by 50%) of cells assessed 24 h after exposure to dFdCyd was significantly lower than the D_{50} of cells assessed immediately after drug treatment (Table 1). This difference in surviving fraction between cells which are assessed immediately versus those that are assessed 24 h after a brief drug exposure is consistent with our previous observations and suggests that immediate trypsinization has a protective effect [19]. We could then ascertain whether, as was the case for radiation [19], noncytotoxic concentrations of dFdCyd could sensitize cells to MMC. We then determined the clonogenic survival of HT29 cells exposed to noncytotoxic dFdCyd (0.1 m*M* for 2 h) with MMC either in the 2nd hour (concurrent) or 24 h after dFdCyd treatment (sequential) (Table 1, Fig. 2). These experiments revealed that unlike radiation, noncytotoxic concentrations of dFdCyd did not enhance MMC cytotoxicity.

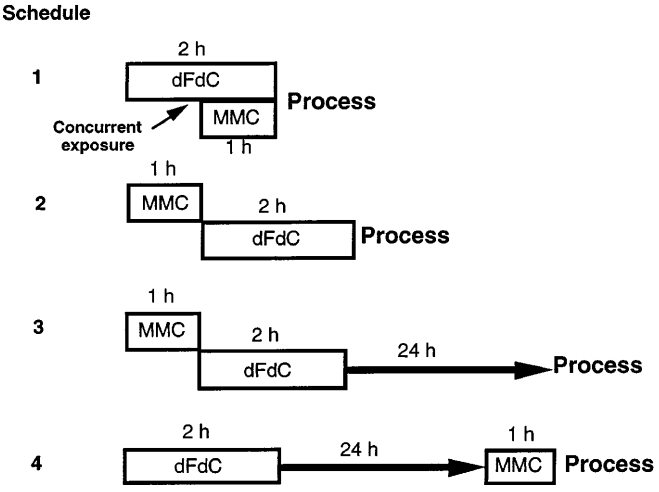

Fig. 2 Experimental design of the study

Since it was possible that synergy required both agents to be used under cytotoxic conditions, we examined different cytotoxic dose ratios of the two drugs using median effect analysis. First, it was necessary to determine whether the relationship between drug concentration and surviving fraction for both the individual drugs and the drugs in combination could be fitted by a sigmoid curve. We found that this was the case (Fig. 3; data not shown for schedules 2, 3, and 4). We were then in a position to determine the dependence of drug interaction on sequence (schedules 1 and 2; Fig. 2). A comparison of these conditions would reveal whether synergy required MMC or dFdCyd to be administered first. Each of the above schedules was investigated using concentration ratios of dFdCyd to MMC of 1:1, 10:1, and 1:10. We found that concurrent exposure (schedule 1) produced CIs consistently below 1 for all concentration ratios. This finding suggests a strong synergistic interaction between the two drugs. On the other hand, MMC followed by dFdCyd (schedule 2) did not show synergy. In fact, the CIs for schedule 2 were suggestive of additivity or even antagonism (CIs greater than 1; Table 2).

When we found that the schedule of MMC followed by dFdCyd did not produce synergy (schedule 2), we assessed the same schedule after a 24-h delay to permit

Table 1 Cytotoxicities of dFdCyd and MMC. Cells were treated with various concentrations of dFdCyd, or with various concentrations of MMC with or without 0.1 μ*M* dFdCyd according to the indicated schedule and assessed for clonogenic survival. The results were fitted to the equation: surviving fraction = $D_{50}^{M}/(D_{50}^{M} + D^{M})$ where D_{50} is the concentration that reduces the surviving fraction to 50%, and *M* is the slope of the sigmoid curve. The area under the cell survival curve was calculated as described in Materials and methods; a smaller area indicates greater drug sensitivity

Drugs	Condition	D_{50} (m*M*)	*M*	AUC
MMC	1 h exposure, process immediately	1.16 ± 0.17	3.88 ± 0.60	1.31 ± 0.16
DFdCyd (various concentrations)	2 h exposure, process immediately	5.84 ± 0.99	1.46 ± 0.66	5.30 ± 0.73
DFdCyd (0.1 μ*M*) + MMC	MMC during 2nd hour of dFdCyd	0.95 ± 0.46	3.24 ± 0.82	1.11 ± 0.45
DFdCyd (various concentrations)	2 h exposure, process 24 h later	0.18 ± 0.05	0.94 ± 0.11	0.71 ± 0.16
DFdCyd (0.1 μ*M*) + MMC	MMC, 24 h after dFdCyd	0.80 ± 0.02	4.09 ± 0.25	0.88 ± 0.01

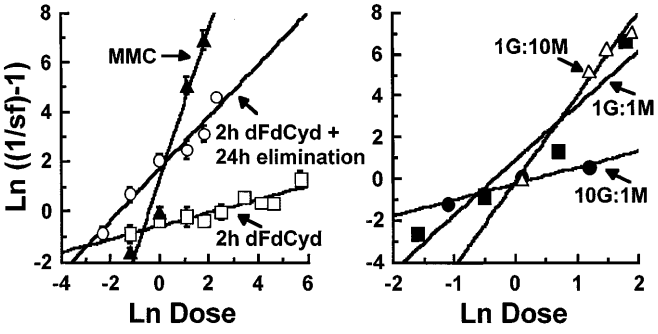

Fig. 3A,B Assessment of appropriateness of median effect analysis for dFdCyd-MMC interactions. **A** Cells were exposed to dFdCyd or MMC, assessed for clonogenicity, and the data were fitted to the equation $\ln(1/(\text{surviving fraction})-1) = m\ln(C)-m\ln(C_{50})$, as described in Materials and methods. **B** Cells were exposed to fixed ratios of dFdCyd and MMC under the conditions described in schedule 1. For example, with a fixed ratio of 1:1, a total concentration of 6 μ*M* indicates 3 μ*M* dFdCyd and 3 μ*M* MMC. The values are the mean ± standard error of at least three experiments. Correlation coefficients for all lines are greater than 0.85

any effects of dFdCyd on the repair of MMC lesions to develop (schedule 3). Evaluation of the CIs resulting from treatment with MMC followed by dFdCyd with delayed processing (schedule 3) produced a complex pattern suggesting, in general, additivity, although it is possible that synergy existed at a ratio of 1:1 (Table 2).

In addition, we wished to assess whether synergy occurred in cells treated with MMC 24 h after dFdCyd treatment (schedule 4). These conditions produce potent radiosensitization of HT29 cells [19]. Unlike the radiosensitization seen previously, we found that this sequence (schedule 4) overall suggested additivity with CIs near 1 (Table 2).

Discussion

In this study, we wished to determine whether combinations of dFdCyd and MMC would be synergistic. A concurrent schedule of dFdCyd and MMC combination produced the largest synergistic effect under these conditions as based on the criteria set by Chou and Talalay [5]. In contrast, exposure to MMC following dFdCyd treatment had either an additive or an antagonistic effect. These findings are consistent overall with those of a previous study investigating the effects of the combination of dFdCyd and MMC against a non-small-cell lung cancer line. In that study, dFdCyd and MMC were synergistic after a 4-h exposure when given at a constant molar ratio (dFdCyd:MMC 1:4), but after a 24–72-h exposure only additivity was produced.

A number of potential mechanisms could explain dFdCyd-MMC synergy. One possibility is that dFdCyd increases MMC crosslinking or decreases crosslink removal. Another possibility is that MMC increases dFdCyd-induced DNA double strand breaks, although this was not found in the study alluded to above [22]. A less likely possibility is that dFdCyd may affect MMC metabolism by modulating a bioreductive pathway (such as DT-diaphorase [30]). Further study will be required to determine which of these and other possibilities is the most likely.

Regardless of the mechanism for dFdCyd-MMC synergy, it is clear that dFdCyd interacts differently with radiation compared to MMC. In contrast to our findings with dFdCyd and MMC, radiosensitization is produced under noncytotoxic dFdCyd conditions and when dFdCyd treatment precedes radiation by 24–48 h. This suggests that the nature of the DNA damage is a key determinant of dFdCyd-mediated sensitization.

dFdCyd has also been examined in combination with cisplatin. A synergistic interaction between the combination of dFdCyd and cisplatin in vitro as well as in vivo in animal studies and also in clinical studies has been reported [4, 23]. However, experimental studies of this in vitro combination have revealed that the synergistic effect is much more pronounced when cisplatin exposure precedes dFdCyd exposure, unlike our findings which showed an opposite effect.

An important issue in interpreting the results of this study concerns the method which we used to assess synergy. Although the median effect principle is a well-established method of assessing drug-drug interactions, other methods, such as dose-surface response [12] and isobologram analysis [31] have also been used. Each approach has its proponents, and it is not clear at this time which of these methods produces more clinically relevant results. In addition, the overall lack of cytotoxicity from dFdCyd alone, at the clinically achievable concentrations that we chose [1], in cells processed immediately after trypsin treatment makes it more difficult to quantify synergy. However, the finding of synergy for

Table 2 Combination indices for all ratios of dFdC:MMC at surviving fraction (*SF*) of 0.1 and 0.03. Cells were treated with various concentrations of dFdCyd and MMC at a fixed ratio of 10:1, 1:1 or 1:10 (dFdCyd:MMC). The combination indices were then calculated, as described in Materials and methods, at a surviving fraction of 0.1 or 0.03

Schedule	SF = 0.1			SF = 0.03		
	10:1	1:1	1:10	10:1	1:1	1:10
1	0.66 ± 0.12	0.49 ± 0.17	0.79 ± 0.14	0.64 ± 0.16	0.78 ± 0.07	0.72 ± 0.12
2	2.75 ± 1.20	1.15 ± 0.12	1.72 ± 0.31	3.43 ± 1.70	1.03 ± 0.15	1.30 ± 0.18
3	0.91 ± 0.10	0.60 ± 0.04	1.42 ± 0.08	0.82 ± 0.07	0.44 ± 0.05	1.08 ± 0.08
4	1.32 ± 0.23	0.92 ± 0.15	1.23 ± 0.40	1.01 ± 0.09	0.61 ± 0.13	1.04 ± 0.1

both the delayed and relatively cytotoxic schedule 3 and the immediate and relatively noncytotoxic schedule 1 suggests that dFdCyd chemosensitization occurs under a range of conditions.

Based on these findings, we have designed a phase I trial of combination MMC and dFdCyd for patients with advanced refractory malignancies. MMC is administered at its usual dose of 10 mg/m^2 every 6-week cycle for two cycles, and dFdCyd is dose-escalated from a starting dose 500 mg/m^2 given weekly for 12 weeks within two cycles of treatment. A total of six patients have been accrued, and dose-limiting toxicity has not yet been reached. Although it is too early to judge the efficacy of this approach, we hope that the antitumor cell synergy observed in vitro will translate to the clinic.

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