Gemcitabine as a Radiosensitizer in Undifferentiated Tumors

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Abstract. *Background: Gemcitabine (dFdC) may cause radiosensitization by specific interference with homologous recombination-mediated DNA double-strand break repair. The radiosensitizing effect of dFdC might be less in normal healthy tissue and more restricted to undifferentiated tumor cells, making it a tumor-selective radiosensitizer. Whether dFdC acts as a radiosensitizer in undifferentiated and well-differentiated rat tumors and on rat foot skin was tested. Materials and Methods: Undifferentiated L44 lung tumors in BN rats, MLL prostate tumors in Copenhagen rats, and well-differentiated L42 lung tumors in WAG/Rij rats were used. The tumors were treated with a single X-ray dose, combined or not with dFdC (30 mg/kg) administered 24 h earlier. Tumor volume growth delay was the end-point used. In addition, rat foot skin was treated with a single dose of 22.5 Gy, with or without dFdC. The degree of skin damage was determined according to a scoring system. Results: For tumor growth delay, the dose-enhancement ratios were 1.37 and 1.23-1.36 for the L44 and MLL tumors, respectively. No radiosensitization was observed for the well-differentiated L42 tumor and foot skin. Conclusion: Radiosensitization by dFdC was observed in the undifferentiated tumors, but not in the well-differentiated tumor and skin. Our data support further trials to evaluate the usefulness of dFdC as a radiosensitizer in undifferentiated tumors.*

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, Gemzar, dFdC) is a deoxycytadine analog well known for its antitumor activity in different tumor types (1-8). It has been shown that dFdC enhanced radiation-induced chromosomal aberrations (9), which suggests interference with repair of DNA damage, particularly the repair of double-strand breaks (DSBs). It was found that homologous recombination (HR) was involved in the synergistic interaction between dFdC and cisplatin (10) and that dFdC causes radiosensitization by specific interference with HR (11). Increased radiosensitivity was observed in HR-deficient irs1 and irs1SF hamster cell lines and in HR-deficient *Drosophila melanogaster* (12). Adult mice deficient in HR, however, did not show hypersensitivity to radiation, and the impact of HR deficiency only became apparent in a non-homologous end joining (NHEJ)-deficient background (13, 14). From these, it was concluded that HR plays a minor role in the repair of double-strand breaks in mature, differentiated cells (11). In undifferentiated cells, HR may contribute to DSB repair and, hence, to cellular radiosensitivity. It was speculated that the radiosensitizing effect of dFdC in patients might be less in normal tissues and more restricted to undifferentiated tumors (11). The radiosensitizing effect of dFdC was reported for several experimental tumors, most of them undifferentiated to poorly-/moderately-differentiated (1-8). In the present study, rats were used to test whether dFdC-mediated radiosensitization occurs in undifferentiated lung and prostate tumors and in a well-differentiated lung tumor. In addition, the responses of the skin as a well-differentiated tissue were scored.

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Materials and Methods

Animal strains and tumors. L44 is a radiation- (external chest irradiation) induced undifferentiated carcinoma, originally diagnosed as an adenosquamous lung carcinoma, which grows in female BN(Orl)Ico rats, (Charles River, Maastricht, The Netherlands) with a tumor volume doubling-time of about 4 days (15-17). The R3327-MATLyLu prostate tumor (MLL) in male Copenhagen rats (Cop/Hsd, Harlan World Head Quarters, Indianapolis, Indiana, USA) is a fast growing anaplastic and metastasizing tumor (18, 19) with a tumor volume doubling-time of about 2 days. L42 is a radiation- (I-125) induced well-differentiated squamous cell carcinoma and grows in female WAG/Rij rats (Charles River) with a tumor volume doubling-time of about 4 days (15-17).

Female BN and WAG/Rij rats and male Copenhagen rats were inoculated in the flank, under isoflurane anesthesia, with tumor pieces of about 2 mm^3.

Table I. *Scoring system of acute skin reactions of rat foot after radiation treatment.*

Symptom	Score
Slight reddening, dry desquamation, or moist desquamation of <25% of sole area	0.5
Reddening, or moist desquamation >25% of sole area	1
Two toes attached	2
Three toes attached	3
Four toes attached	4
Club-foot	5

Score can be higher by combination of symptoms

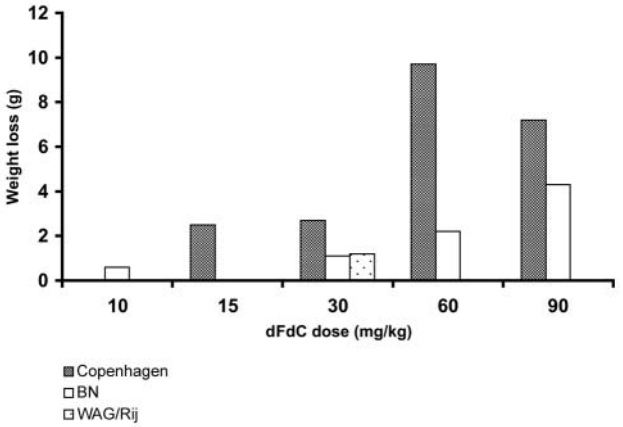

Figure 1. *Maximum weight loss of Copenhagen, BN and WAG/Rij rats after administration of a single dose of dFdC.*

Treatment. DFdC (Eli Lilly, Nieuwegein, The Netherlands) was reconstituted in physiological saline and stored at –30˚ C. The drug was injected *i.p.* into rats at room temperature using single doses ranging from 10 to 90 mg/kg in a volume of about 2 to 3 ml. The weight of the animals at the start of the treatments was about 170 g (BN and WAG/Rij rats) and 220 g (Copenhagen rats).

X-ray doses (200 kV, 20 mA, 0.5 mm Cu, dose-rate 4 Gy/min, Philips Orthovolt RT250) were administered locally under hypnorm/dormicum anesthesia. When combined with dFdC at a concentration of 30 mg/kg, irradiation was performed 24 h after dFdC administration. The time-interval of 24 h was chosen based on data in the literature (2, 3).

Tumors were treated at a volume of about 0.5 - 1 cm^3. L44 tumors were treated with a single dose of 10 Gy, MLL tumors with single doses of 20 and 30 Gy and L42 tumors with a single dose of 15 Gy, with or without dFdC. The tumors were measured twice per week with calipers. The tumor volume was based on 2 orthogonal cross-sectional diameter measurements ($V=0.5a^2b$ with a the smallest diameter). The volumes were then expressed as a percentage of the pretreatment volume on day 1, which was designated as 100%.

Skin. Early skin responses of a hind foot following irradiation with a single dose of 22.5 Gy, with or without dFdC, were scored over a period of 5 weeks. The degree of damage as a function of time after treatment was determined according to the scoring system shown in Table I.

End-points. Excess growth delay (EGD) is the time-interval for the tumor to reach 4 times its pre-treatment volume (T4t) minus the time for a control tumor (with the same volume as the pre-treatment volume of the treated tumor) to reach 4 times that volume (T4): EGD = T4t-T4. The specific growth delay (SGD) was calculated for each treated tumor: SGD = EGD/T4. Using SGD, differences in the growth rate of tumors differing in starting volume at day 0 and between experiments can be dealt with. For skin, the mean value and standard error of the maximal skin scores of animals at risk per experimental group were determined. The enhancement ratio is defined as SGD(radiation+dFdC)/ SGD(radiation).

Statistics. The Kaplan-Meier analysis was performed using SPSS10.1 by scoring an event as EGD, SGD, or maximum skin score.

Approval. The Animal Experiments Ethical Committee of the University Medical Center Utrecht, The Netherlands, approved the animal experiments.

Results

Toxicity of dFdC. Single doses of dFdC, ranging from 10 to 90 mg/kg, were administered *i.p.* to tumor-bearing animals. The weight of the animals and tumor volumes were recorded. The maximal mean weight loss of BN rats for dFdC doses from 10 to 90 mg/kg was 0.6 to 4.3 g (0.9 to 2.5%); for Copenhagen rats for dFdC doses from 15 to 90 mg/kg, this value was in the range of 2.5 to 9.7 g (1.1 to 4.4%); for Wag/Rij rats it was 1.3 g (0.8%) for a dose of 30 mg/kg, Figure 1.

dFdC, tumors. For a dFdC dose of 30 mg/kg, the mean and standard error (SE) of the EGD and mean and SE of the SGD for L44 tumors in BN rats were 1.6±0.4 days and 0.24±0.06, respectively. For MLL tumors in Copenhagen rats, these values were 1.1±0.7 days and 0.3±0.2, respectively, and for L42 tumors in Wag/Rij rats were 10.4±2.7 days and 0.78±0.21, respectively. For the experiments to determine whether dFdC would interact with radiation, the dose of 30 mg/kg dFdC was selected, *i.e.* a dose with a small effect on weight loss and tumor growth delay.

dFdC and radiation, tumors. L44 tumors were treated with a single dose of 10 Gy, resulting in a mean SGD of 1.53 (1.19-1.87). For the combined treatment of dFdC (30 mg/kg) and a dose of 10 Gy, the SGD = 2.09 (1.86-2.33). The enhancement ratio (ER) was 2.09/1.53 = 1.37±0.23 (Table II).

MLL tumors were treated with single doses of 20 and 30 Gy, and the results are shown in Table II. The ER were

Table II. *Specific growth delay (SGD) for 3 rat tumors with 95% confidence interval (95% CI) for dFdC, single radiation doses and combined treatments, with enhancement ratio (ER)±standard error (SE); N, number of tumors tested.*

Tumor	Treatment	N	SGD (95% CI)	ER±SE
L44	dFdC (30 mg/kg)	11	0.24 (0.13-0.35)	
	10 Gy	15	1.53 (1.19-1.87)	
	dFdC + 10 Gy	15	2.09 (1.86-2.33)	1.37±0.23
MLL	dFdC (30 mg/kg)	10	0.32 (0-0.69)	
	20 Gy	5	2.91 (1.87-3.95)	
	dFdC + 20 Gy	5	3.97 (3.43-4.51)	1.36±0.34
	30 Gy	10	3.55 (3.17-3.94)	
	dFdC + 30 Gy	8	4.37 (3.33-5.40)	1.23±0.22
L42	dFdC (30 mg/kg)	14	0.78 (0.37-1.19)	
	15 Gy	13	3.73 (3.30-4.17)	
	dFdC + 15 Gy	19	3.73 (3.45-4.01)	1.0±0.1

dFdC=gemcitabine

1.36±0.34 and 1.23±0.22 for the combinations dFdC + 20 Gy and dFdC + 30 Gy, respectively.

L42 tumors were treated with single doses of 15 Gy. The results are shown in Table II. The ER was 1.0±0.1. Although the L42 tumors were more affected by the dFdC dose than the other 2 tumors (SGD = 0.78 *versus* 0.24 and 0.32), the combined effect of dFdC and radiation did not show any radiosensitization by dFdC at all.

Examples of growth curves of the 3 tumors and responses to treatments are shown in Figure 2.

dFdC and radiation, skin. For BN rats at the dose of 22.5 Gy, the mean skin score was 2.9±0.7 and for the combination of dFdC (30 mg/kg) and 22.5 Gy, the mean score was 2.5±0.7. For Copenhagen rats the mean scores were 4.5±0.7 and 3.7±0.7, while these values were 2.9±0.9 and 2.75±0.3, respectively, for WAG/Rij rats. Surprisingly, the mean scores after the combination treatment were less than after radiation only, indicating radioprotection by dFdC. However, these differences are not statistically significant. The mean maximum skin scores and 95% confidence intervals for the 3 rat strains are summarized in Table III.

Discussion

In our experiments, it was found that dFdC enhanced the radiation response in 2 undifferentiated rat tumors, but not in the well-differentiated L42 tumor or rat skin. This is in accord with the expectation that a radiosensitizing effect of dFdC might be present in undifferentiated tumors.

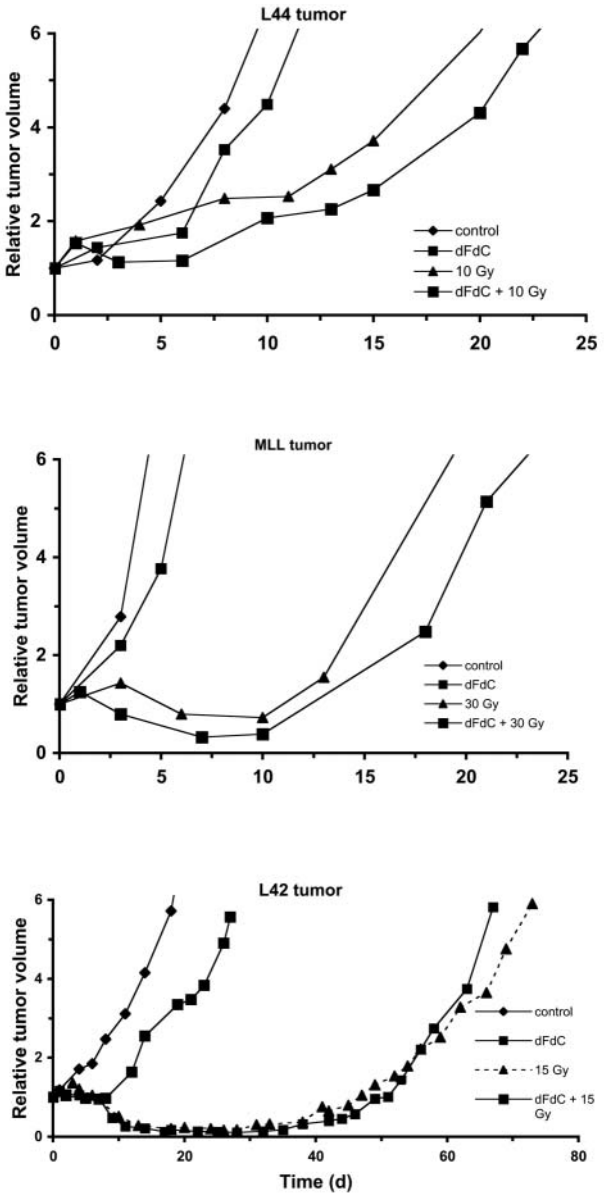

Figure 2. *Growth curves of control L44, MLL and L42 tumors, and curves after start of treatment with dFdC, a single dose of X-rays and combined treatments.*

Evidence suggests that the radiosensitization effect of dFdC is associated with a redistribution of cells into the S-phase with a simultaneous depletion of dATP pools (20-22). Others reported that dFdC is an effective inhibitor of DNA synthesis (23-25) and inhibits the repair of radiation-induced chromosome damage *in vitro* (26).

Experiments have also addressed the possibility that reoxygenation of hypoxic cells is an additional mechanism by which dFdC enhances tumor radioresponse (2). In addition, the elimination of the S-phase cells from the

Table III. *Mean scores with standard errors (SE) and 95% confidence intervals (CI) of foot skin reactions of 3 rat strains after treatments with a single dose of 22.5 Gy, and a dose of 22.5 Gy, 24 h after administration of dFdC (30 mg/kg) (dFdC+ 24 h + 22.5 Gy).*

	Mean skin score					
	22.5 Gy			dFdC + 24 h + 22.5 Gy		
Rat strain	N	Mean±SE	95% CI	N	Mean±SE	95% CI
BN	17	2.9±0.7	(1.5 – 4.2)	15	2.5±0.7	(1.2 – 3.8)
Cop/Hsd	16	4.5±0.7	(3.2 – 5.8)	15	3.7±0.7	(2.4 – 5.1)
WAG/Rij	5	2.9±0.9	(1.3 – 4.4)	4	2.7±0.3	(2.3 – 3.2)

dFdC=gemcitabine

tumor population by dFdC and the redistribution of surviving cells into a more radiosensitive compartment of the cell cycle may play a part (2). The target for radiosensitization induced by dFdC was found to be homologous recombination (HR) of DNA double-strand breaks (11) rather than the non-homologous end-joining pathway (27). As was demonstrated by Essers *et al.* (14), HR-deficient mice are hypersensitive to ionizing radiation at the embryonic, but not at the adult stage. Thus, a defect in HR may affect the radiosensitivity of undifferentiated embryonic stem cells (13) and cultured cell lines (28-37) and its impact on the radiosensitivity of differentiated adult cells *in vivo* might be limited or even absent (11). Wachters *et al.* (11) speculated that the radiosensitizing effect of dFdC in patients might be less in normal healthy tissue and more restricted to (undifferentiated) tumor cells, making it a tumor-selective radiosensitizer. Therefore, it was of interest to study the responses of both un- and well-differentiated experimental tumors to dFdC and to ionizing radiation as well as the responses of the normal, well-differentiated, tissue skin. Our results indicate that the expectation of Wachters *et al.* might be true.

Radioenhancement in experimental tumors. Several authors (1-8) reported the enhancement of radiosensitivity by dFdC in experimental tumors. We expected that radio-enhancement primarily occurs in undifferentiated and poorly-differentiated tumors. The tumor models showing radioenhancement are detailed in Table IV. The human squamous carcinoma FaDu (1) and pancreatic tumor MiaPaCa-2 (8, 38), the mouse SA-NH sarcoma (2, 3, personal communication) and the SCC VII squamous cell carcinoma (4, 39) all are undifferentiated or poorly-differentiated. The mouse mammary adenocarcinoma CH3/TIF (6, 7) and the human BxPC-3 pancreatic tumor (8, 40) are poorly- to moderately-differentiated. The mouse hepatocarcinoma Hca-I might be a well-differentiated tumor (personal communication, 2004) and may be an exception. However, this tumor was described as "looking like a well-differentiated tumor", leaving other possibilities open. In all these tumors dFdC enhanced the radioresponsiveness.

The enhancement factors described were 1.6 to 3.3 for the human squamous carcinoma FaDu, depending on the dFdC dose and administration schedule (1) and more than 3 for the MiaPaCa-2 xenograft (8). For the SA-NH tumors the enhancement factors were 1.54 to 2.03, also depending on the time-interval between dFdC and radiation administration and end-point (cure, growth delay) (2), and 1.34 to 1.46 for growth delay (3). Fields *et al.* (4) reported an enhancement ratio of 1.6 to 1.8 for the SCC VII squamous tumor. An enhancement ratio larger than 2 for the BxPC-3 xenograft could be derived from the experiments described by Buchsbaum *et al.* (8) and Cividalli *et al.* (7) who reported an enhancement ratio of 1.55 to 1.96 for single dose irradiation and 2.17 to 4.86 for fractionated irradiation. The ER was 1.6 for the hepatoma (5). These dose schedules and enhancement ratios are summarized, in Table IV, from which it is clear that our dFdC dose of 30 mg/kg was relatively low. Only in the experiments of Mason *et al.* and Joschko *et al.* (1, 3) were comparable dose levels used.

The response of the MLL tumor to combined treatment – ER = 1.23 to 1.36 – may be somewhat less than that of the L44 tumor with an ER of 1.37, depending on the radiation dose. This may be caused by the inappropriate vasculature of the tumor, preventing dFdC from adequately reaching all tumor cells. In earlier experiments, interaction with the radiosensitizer Motexafin was not observed (41). For our L42 tumor, no enhancement at all could be found.

The specific growth delays after combined treatment in the L44 and MLL tumors were larger than the sum of the SGDs of the treatments with dFdC or radiation only (Table II). In contrast, although the L42 tumors were more affected by the single dFdC dose than the other 2 tumors, the combined effect of dFdC and radiation did not show any radiosensitization by dFdC at all.

Table IV. *Histological characteristics of experimental tumors and enhancement ratios.*

Tumor	Origin	Strain	Histology	Schedule dFdC and radiation	Enhancement ratio	Reference
		Mice				
FaDu	Human squam carc.	Balb/c nude	Poorly- to moderately-diff.	10 x 2.3 mg/kg daily + 10 x (2 x 2 Gy)	1.6	(1)
				once weekly, 2 x 430 mg/kg + 10 x (2 x 2 Gy)	2.6	
				twice weekly, 4 x 50 mg/kg + 10 x (2 x 2 Gy)	2.4	
				twice weekly, 4 x 160 mg/kg 10 x (2 x 2 Gy)	3.3	
MiaPaCa-2	Pancreatic carc.	Balb/c nude	Undiff.	6 x 120 mg/kg + 6 x 3 Gy	>3	(8,38)
Sa-NH	Sarcoma	C3Hf/Kam	Poorly-diff.	50 mg/kg + 25 Gy, time-interval	1.68 – 2.03	(personal communication)
				50 mg/kg + graded doses (cure)	1.54	(2)
				5 x 3 Gy, 5 x 5 Gy or 5 x 7 Gy + 1 x 25, 2 x 12.5 and 5 x 5mg/kg	1.34 –1.46	(3)
SCC VII	Squamous carc.	C3H	Poorly-diff.	1 x 800 mg/kg + 5 x 5.5 Gy daily	1.6	(4)
				2 x 100 mg/kg + 5 x 5.5 Gy	1.8	
BxPC-3	Pancreatic adenocarc.	Balb/c nude	Poorly- to moderately-diff.	6 x 120 mg/kg + 6 x 3 Gy	>2	(8,40)
CH3/TIF	Mammary adenocarc.	C3D2F1	Moderately-diff.	60/120 mg/kg + 10 Gy	1.76 /1.66	(6,7)
				60/120mg/kg + 20 Gy	1.55 / 1.96	
				4 x 60 mg/kg + 10 x 2 Gy	4.86	
				4 x 60 mg/kg + 4 x 10 Gy	2.17	
Hepatoma	Hepatocarc.	C3H/HeL	"Looks like a well-diff. tumor."	50 mg/kg + 25 Gy	1.6	(5, personal communication)
		Rats				
L44	Adenosquam. carc.	BN	Undiff.	30 mg/kg + 10 Gy	1.37	present
MLL	Prostate cancer	Cop/Hsd	Undiff.	30 mg/kg + 20 or 30 Gy	1.36 / 1.23	present
L42	Squamous carc.	WAG/Rij	Well diff.	30 mg/kg + 15 Gy	1.0	present

Normal tissues. We did not find any radioenhancement by dFdC (30 mg/kg) in the rat foot skin; on the contrary, some protection was observed. This effect was also reported for a nude mice model by Classen *et al.* (42) who found that acute and late toxicity of skin and underlying soft tissues of the hind leg of NMRI-nu/nu-nude mice was not significantly increased after single-dose irradiation in combination with dFdC (550 mg/kg) with a time-interval of –36 to +24 h. Even a slight radioprotective effect for dFdC was suggested. Cividalli *et al.* (6), in their study of acute skin reactions in mouse hind leg, reported that the addition of dFdC to radiation did not, in any case, modify the results. The response of the jejunum of C3Hf/Kam mice with the microcolony assay was strongly dependent on the schedule of dFdC administration, single dose of 25 mg/kg, 2x12.5 mg/kg or 5x5 mg/kg. A slight radioprotection to enhanced radiation response, ranging from 0.96 to 1.23 (3), was observed. Gastrointestinal toxicity was also investigated by Gregoire *et al.* (9), who applied whole-body irradiation. Depending on the time-interval between dFdC administration (150 mg/kg) and irradiation, an enhancement ratio of 0.9 to 1.3 was observed. These ratios are quite similar to those found by Mason *et al.* (3). These results indicate that the enhancement ratios observed are not related to the relatively high dFdC concentrations. An increase in oral mucosa reaction of C3H mice was observed with the combination of 5 daily fractions of 5.5 Gy and dFdC administered as a single dose of 800 mg/kg or 2x100 or 2x150 mg/kg *versus* radiation only (4). From the figures shown by Fields *et al.* (4), it can be deduced that the combined treatment (27.5 Gy and dFdC) is equivalent to radiation alone at about 29 Gy. The enhancement ratio thus is about 1.05. However, the dFdC doses alone produced a weight loss of about 10% and were much higher than in our experiments or in those of Mason *et al.* (3). Gregoire *et al.* (43) also studied the effect of

Table V. *Enhancement ratio (ER) of some normal tissues.*

Tissue	Strain	dFdC (mg/kg)	Interval* (h)	ER	Reference
Skin	NMRI-nu/nu	550	36 – 2; 24 h after irr.	ns	(42)
Skin	C3D2F1	30, 60, 120	24	1	(6)
Jejunum	C3Hf/Kam	25	24	0.96 – 1.23	(3)
Jejunum	C3H	150	3 – 48	0.9 – 1.3	(9)
Oral mucosa	C3H	800	6	1.05	(4)
		2x100/150		1.05	(4)
Lung	C3H	150	3 - 48	1.1	(43)
Skin	BN, Copenh WAG/Rij	30	24	<1	present

dFdC=gemcitabine
*interval between dFdC and irradiation

dFdC (150 mg/kg) on the tolerance of the lung to single-dose irradiation in C3H mice. The time-interval between dFdC and irradiation varied from 3 to 48 h. Their data indicated a minimal effect of dFdC on lung tolerance after irradiation. The LD_{50} values for the combination were reduced by about 10%.

From this short review on normal tissue tolerance, (Table V), we may conclude that a slight protection to enhanced radiation response was observed for normal tissues, depending on the treatment schedule, dosage of dFdC and end-point. The observed enhancement ratios were, in general, less by up to 1.3 than those observed for the undifferentiated to poorly-/moderately-differentiated tumors, ranging from 1.23 to greater than 4, (Table IV), hence indicating a therapeutic gain. These findings support the concept of using full-dose radiation and of attempting to improve local control with dFdC applied at a relatively low dose as a radiosensitizing agent.

Conclusion. Radiosensitization due to a relatively low dose of dFdC was observed in the undifferentiated L44 and MLL tumors, but not in the well-differentiated L42 tumor or skin. Our data support further trials to evaluate the usefulness of dFdC as a radiosensitizer for undifferentiated tumors.

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