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Effect of Scheduling of Combinations of 5-(3,3-Dimethyl-1-triazeno)-imidazole-4-carboxamide and 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea on the Harding-Passey and Cloudman S91 Mouse Melanomas[1]

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ABSTRACT

Harding-Passey mouse melanoma (HP) cells (10^6) were administered i.p. to female BALB/c × DBA/2 F_1 (hereafter called $CD2F_1$) mice on Day 0. We showed earlier (H. Z. Hill, *et al.*, Arch Surg., *114:* 135–138, 1979) that a single dose of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) administered on Day 0 or on subsequent days was equally effective regardless of the day of administration. We now show that a single dose of 10 mg of 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU) per kg was most effective in prolonging survival of HP-bearing $CD2F_1$ mice when administered on Day 0. There was then a decline in effectiveness to Day 10, at which time the increase in survival of the drug-treated animals was no longer significant.

The effect of sequential scheduling of DTIC and MeCCNU on HP was studied. The first drug was given on Day 0 or on Day 10. The second drug followed on the same day or on subsequent days. The greatest enhancement of survival was seen when DTIC was administered on Day 0 and MeCCNU on Day 1. Nine of 10 mice on this schedule were cured. When treatment of HP was started on Day 10, the most enhancement was again seen for DTIC on Day 10 and MeCCNU on the next day. Reversal of the order of the two drugs produced less prolongation of survival and fewer cures.

The effect of scheduling two doses of DTIC was also studied using the HP model. The first dose was given on Day 0 or on Day 10. The second dose produced the greatest enhancement of survival when administered 3 to 4 days after the first dose, but the enhancement was less than that seen when DTIC was followed by MeCCNU.

For comparison, the two drugs were also studied in female DBA/2 mice carrying the Cloudman S91 melanoma. In combination, on Day 0, in only one of three experiments was survival prolonged beyond the controls. Other schedules were ineffective. The enhancement seen when HP-bearing $CD2F_1$ mice are treated with the best combination of the two drugs is clearly not seen with S91. The results imply that dosage scheduling in the treatment of murine melanomas must be individualized. Extrapolation to the human situation suggests the same conclusion.

INTRODUCTION

The aim of our study is to devise better treatment modalities for melanoma. We are currently investigating the effects of combinations of drugs which are used in the treatment of human melanoma (7). Our approach is (*a*) to study only two drugs at a time; (*b*) to simplify the protocol as much as possible by giving only one injection of each drug (the extent of cell kill by single doses is directly related to the MST[3] as shown previously); (*c*) to use minimally effective doses of the drugs as single agents so that marked interactions (hopefully cures) will be easily detected; and (*d*) to study different representatives of same tumor type in the hope of uncovering generalizations. The drugs we have chosen to investigate initially are DTIC, MeCCNU, and L-PAM. We are studying the effects of scheduling these drugs 2 at a time using 3 transplantable mouse melanomas: B16 melanoma grown in female C57BL/6 mice; HP, grown in female BALB/c × DBA/2 F_1 (hereafter called $CD2F_1$) mice; and Cloudman S91 melanoma grown in female DBA/2 mice. In our first report (8), we determined the doubling times of the 3 tumors and the effects of the agents administered singly. In our second report (9), we studied the effect of combinations of DTIC and L-PAM against HP. We found that there was marked increase in survival when DTIC was administered first, followed by L-PAM administered on the following day. This effect was not observed in B16 melanoma. In the present report, we describe our studies of dosage scheduling using DTIC and McCCNU against HP and Cloudman S91 tumor. In these experiments on combinations, we have generally used doses which are below toxic levels for single agents. The Southern Research Institute reports an LD_{10} for DTIC of 374 to 517 mg/kg for 3 mouse strains and for MeCCNU of <18 to 35 mg/kg for 6 mouse strains.[4]

MATERIALS AND METHODS

Tumors. The tumors and the methods of passage were described previously (8, 9). For experiments, single-cell suspensions of tumor cells were injected i.p. into mice on Day 0. HP was injected at 10^6 viable cells/mouse and S91 was injected at 2×10^6 viable cells/mouse. Treatment cages contained 5 mice each. Fifteen no-drug control mice were used in all experiments.

Drugs. Drugs were prepared in solution daily and were administered i.p. DTIC was supplied by Dome Laboratories, West Haven, Conn., and was dissolved in 1% citric acid: 0.5% mannitol. MeCCNU was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. It was dissolved in

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[3] The abbreviations used are: MST, median survival time; DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; MeCCNU, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; L-PAM, L-phenylalanine mustard; HP, Harding-Passey melanoma; LD_{10}, 10% lethal dose; CLS, change in life span.

[4] Southern Research Institue. LD_{10} Summary, 1975.

polyoxyethylated vegetable oil (Emulphor EL-620; GAF Corp., New York, N. Y.) at 25 mg/ml. Subsequent dilutions were made in 0.9% NaCl solution. DTIC, at the single doses used in these experiments, caused no deaths in tumor-free female $CD2F_1$ mice. We found that the LD_{10} in 30 tumor-free female DBA/2 mice was 360 mg/kg. MeCCNU is also nonlethal for tumor-free $CD2F_1$ mice at the doses used. The response of tumor-free female DBA/2 mice to MeCCNU was complex, but 25 mg/kg was consistently not lethal. DTIC at 100 mg/kg and MeCCNU at 10 mg/kg administered simultaneously caused no deaths in tumor-free DBA/2 mice.

Calculations. The percentage of CLS was calculated by dividing the MST of a given treatment cage by the MST of the untreated controls. CLS was used instead of increase in life span because some regimens decreased survival. The additive level was determined by adding the CLS of the mice given DTIC alone on Day 0 to the CLS of the mice given MeCCNU alone on Day 0.

RESULTS

Effect of Treatment Day on Survival of HP-bearing Mice. In our earlier study (9) with DTIC and L-PAM using HP, we found that DTIC administered on Day 0, followed by L-PAM on Day 1, produced the greatest enhancement of survival. We also found that L-PAM given alone on Day 1 or Day 2 was more effective than when given at a later time, whereas the response of HP to DTIC alone was not affected by the day of administration.

The effect of the day of treatment of 10 or 15 mg/kg of MeCCNU on HP is shown in Table 1. MeCCNU is more effective when administered on Day 0 than when administered on subsequent days, and there is a decrease in effectiveness with some fluctuations as the time between the injection of the tumor cells and the administration of the drug is increased. When a similar experiment was performed using MeCCNU at 5 mg/kg, there were no significant increases in survival over the controls even on Day 0.

Effect of Scheduling Combinations of DTIC and MeCCNU on HP. Mice were given tumor cells on Day 0 followed by a single injection of either DTIC or MeCCNU on Day 0. The injections of DTIC on Day 0 were followed by single injections of MeCCNU on Day 0 or on subsequent days. A similar regimen was followed for MeCCNU on Day 0, with DTIC on Day 0 or on subsequent days.

HP exhibited a schedule-dependent synergistic response to DTIC and MeCCNU, as seen in Table 2. Here, the most effective dose schedule was DTIC administered on Day 0 and MeCCNU administered on Day 1. In this case, 9 of 10 mice receiving this schedule survived beyond 120 days. These mice had turned gray before the experiment was terminated. The normal coat color of the $CD2F_1$ mouse is agouti. In survivors, it was considerably lighter than normal and had a grayish-brown color. Long-term survivors in other treatment cages in these experiments did not exhibit any change in coat color, suggesting that the most effective schedule also has some specific effect on pigment cells. In order to see if the same effects would be reproducible when lower doses of DTIC and MeCCNU were used, the doses for each drug were reduced by 50%. The synergistic effect on survival as a function of scheduling now disappeared (data not shown). Survival was additive when the second drug was given early, and less than additive when it was given later.

The scheduling regimen that we have chosen is artificial in the sense that therapy is initiated within 2 to 3 hr after tumor cell injection into the peritoneal cavity. At this time, the cells have been shocked by removal from s.c. tumors and resuspension in medium. Furthermore, they are dispersed singly or in small groups throughout the peritoneal cavity and are devoid of a blood supply. This situation bears little resenblance to established tumors, although it may be more sensitive for detecting drug interactions. In order to determine whether the effects that we observed were applicable to established tumors, treatment was started on Day 10 rather than on Day 0. In this case, again, the best scheduling was seen when DTIC was administered first followed by MeCCNU 1 day later (Table 3). In the first experiment with established tumors, the dose of DTIC was 100 mg/kg and the dose of MeCCNU was 10 mg/kg. In the second and third experiments, we increased the dose of DTIC to 150 mg/kg and of MeCCNU to 15 mg/kg. There is no time dependence of DTIC alone at 150 mg/kg (9), and the time response of MeCCNU at 15 mg/kg is shown in Table 1. Mice that received the increased doses of the 2 drugs on the same day died before the controls. However, overall, MeCCNU given 1 or 2 days after DTIC again produced the best survival. The reverse regimen was also more effective and over a longer interval, but there were no long-term survivors in any of these cages, again showing that the best schedule is DTIC

Table 1
Effect of MeCCNU on survival of HP-bearing $CD2F_1$ mice

	Experiment 1			Experiment 2					
	10 mg/kg			10 mg/kg			15 mg/kg		
Day of drug	MST (days)	CLS (%)	S_{120}[a]	MST (days)	CLS (%)	S_{120}	MST (days)	CLS (%)	S_{120}
0	69.5	+271	2/5	46.5	+66	1/5	>120	>327	5/5
1	39.5	+111	0/5	36.8	+31	0/5	69.5	+130	2/5
2	31.5	+68	0/5	33.5	+19	0/5	54.5	+94	1/5
3	32.8	+75	0/5						
4				29.8	+6	0/5	57.5	+105	2/5
5	29.3	+56	0/5						
7	29.8	+59	0/5	33.5	+19	0/5	37.5	+33.6	0/5
10	21.5	+15	0/5	35.8	+28	1/5	60.5	+116	0/5
14				38.5	+37	0/5	42.5	+51	0/5
20				26.8	−5	0/5	34.3	+22	0/5
No-drug controls	18.8		0/15	28.1		0/15	28.1		0/15

[a] S_{120}, number of mice surviving to Day 120 after tumor injection.

Table 2

Effect of combinations of DTIC and MeCCNU on HP-bearing CD2F$_1$ mice: early tumors; DTIC, 100 mg/kg; and MeCCNU, 5 mg/kg

				Experiment 1			Experiment 2		
1st drug	Day	2nd drug	Day	MST (days)	CLS (%)	S_{120}[a]	MST (days)	CLS (%)	S_{120}
DTIC	0			28.2	+28.8	0/5	20.5	−1.4	0/5
	0	MeCCNU	0	74.2	+239	0/5	35.5	+71	0/5
	0		1	>120	>448	5/5	>120	>477	4/5[b]
	0		2	42.5	+94	0/5	39.5	+90	0/5
	0		3	27.8	+27	0/5	34.3	+65	0/5
	0		4	29.3	+34	0/5	25.9	+25	1/5
	0		7	28.5	+30	0/5	22.5	+8	0/5
	0		10	27.5	+26	0/5	26.5	+27	0/5
	0		14	34.8	+59	0/5	27.8	+34	0/5
MeCCNU	0			34.8	+59	0/5	31.5	+51	0/5
	0	DTIC	0	51.5	+135	0/5	37.5	+80	0/5
	0		1	94.5	+332	2/5	35.9	+73	1/5
	0		2	50.5	+131	0/5	42.5	+104	0/5
	0		3	41.5	+89	1/5	36.5	+75	0/5
	0		4	48.5	+121	1/5	46.5	+124	0/5
	0		7	48.2	+120	0/5	36.3	+75	0/5
	0		10	43.5	+99	0/5	24.5	+18	0/5
	0		14	38.5	+76	0/5	34.6	+66	0/5
		No-drug controls		21.9		0/15	20.8		0/15
		Additive level		41.1	+88		31.2	+50	

[a] S_{120}, number of mice surviving to Day 120 after tumor injection.
[b] Autopsy showed no sign of tumor in the mouse that died.

Table 3

Effect of combinations of DTIC and MeCCNU on HP-bearing CD2F$_1$ mice: established tumors; DTIC, 100 mg/kg; MeCCNU, 10 mg/kg (Experiment 1); and DTIC, 150 mg/kg; MeCCNU, 15 mg/kg (Experiments 2 and 3)

				Experiment 1			Experiment 2			Experiment 3		
1st drug	Day	2nd drug	Day	MST (days)	CLS (%)	S_{120}[a]	MST (days)	CLS (%)	S_{120}	MST (days)	CLS (%)	S_{120}
DTIC	10			22.8	+13	0/5	34.5	+13	0/5	34.5	+37	0/5
	10	MeCCNU	10	48.5	+140	0/5	15.5	−49	0/5	21.5	−15	0/5
	10		11	68.5	+239	2/5	80.5	+165	2/5	>120	>376	3/5
	10		12	42.5	+110	0/5	82.5	+171	2/5	82.5	+227	2/5
	10		13	32.8	+62	0/5	85.5	+181	2/5	59.5	+136	0/5
	10		14							59.5	+136	0/5
	10		17							54.5	+116	0/5
MeCCNU	10			31.5	+56	0/5	41.5	+37	0/5	50.8	+102	0/5
	10	DTIC	10	47.5	+135	0/5	19.3	−37	0/5	21.5	−15	0/5
	10		11	39.5	+96	0/5	64.5	+112	0/5	68.5	+172	0/5
	10		12	37.5	+86	0/5	64.5	+112	0/5	68.5	+172	0/5
	10		13	39.5	+96	0/5	72.5	+138	0/5	57.8	+129	0/5
	10		14							67.5	+168	0/5
	10		17							67.8	+169	0/5
	No-drug controls			20.2		0/15	30.4		0/15	25.2		0/15
	Additive level			34.1	+69		45.6	+50		60.1	+138	

[a] S_{120}, number of mice surviving to Day 120 after tumor injection.

first followed by MeCCNU.

Effect of Scheduling 2 Doses of DTIC on HP. It was thought that DTIC might cause some perturbation in the HP cells to make them more sensitive to any agent following at the optimum time, especially since DTIC on Day 0 followed by L-PAM on Day 1 produces a similar enhancement of survival in HP-bearing mice (9). We therefore investigated the effect of scheduling using 2 doses of 100 mg/kg each of DTIC with treatment initiated on either Day 0 or Day 10. The marked enhancement seen in our other experiments by a second drug given 24 hr after the administration of DTIC was not seen in these experiments, in which the second drug administered was a second dose of DTIC, although the best spacing of the 2 doses would appear to be 3 to 4 days (data not shown). There were no long-term survivors in these experiments.

Effects of DTIC and MeCCNU on S91-bearing DBA/2 Mice. The combination of DTIC and MeCCNU was studied in S91. DTIC was given at 100 mg/kg and MeCCNU at 10 mg/kg with therapy started on Day 0. In this case, neither drug prolonged survival at the dose given, when given alone on Day 0. When the 2 were given together on Day 0, survival was prolonged 40 to 50% in one experiment, but when the time between the drugs was increased, survival was not prolonged (data not shown). In 3 other experiments, the combination of DTIC and MeCCNU was ineffective on any schedule. The enhancement observed in HP clearly did not occur in S91.

DISCUSSION

If a drug kills a constant fraction of cells, then the increase in MST should correlate with the fraction of cells surviving. DTIC given to HP-bearing mice produces the same increase in

life span regardless of when it is injected following tumor cell injection. It, therefore, appears that a constant fraction of cells is killed by a single dose of DTIC regardless of when it is administered after tumor injection. MeCCNU is progressively less effective when given at later times to HP-bearing mice. MeCCNU, therefore, appears to kill a progressively smaller fraction of cells as the time after tumor cell injection increases.

The interaction between DTIC and MeCCNU in the HP system bears some resemblance to the interaction between DTIC and L-PAM which we reported earlier in this tumor system (9). When DTIC is given first followed by either L-PAM or MeCCNU 1 day later, there is marked enhancement of survival and most of the mice are cured. As the time between the 2 drugs is increased, the effectiveness of the combination diminishes and the enhancing impact of the second drug becomes progressively less. The increase in survival declines, approaching that produced by DTIC administered alone on Day 0. When the order of the drugs is reversed, the enhanced survival at a spacing of 1 day is not as great as when DTIC is given first. The MST declines less precipitously and again approaches that produced by either MeCCNU or L-PAM alone. The DTIC-MeCCNU interaction is seen even if the combination therapy is started after the tumors have become established, but higher doses are needed to achieve the same effect. DTIC and L-PAM do not exhibit enhancement when used in the treatment of established tumors at doses that are effective on Day 0.[5] We are currently studying the effect of higher doses at later times.

The mechanism of action of DTIC has been studied in several laboratories (2, 5–7, 10–12, 14). Light converts DTIC to diazoimidazole carboxamide which inhibits DNA synthesis in bacteria but is probably not the active metabolite (2). Liver microsomes convert DTIC to aminoimidazole carboxamide, (5, 10) which in ribotide form is an intermediate in purine biosynthesis, and to a precursor of methyl carbonium ion which could then alkylate macromolecules. DTIC in tissue culture has a greater inhibitory effect on RNA and protein synthesis than on DNA synthesis (2). Saunders and Chao (14) believe that dimethylamine may be the active intermediate, while Mizuno's group (11, 12) believes that 5-(3-methyl-1-triazeno) imidazole-4-carboxamide is the principal active intermediate. This latter compound inhibits [^{3}H]thymidine incorporation more than [^{3}H] uridine incorporation, and guanine and adenine in DNA are methylated. 5-(3-Methyl-1-triazeno)imidazole-4-carboxamide-treated DNA has impaired template activity for RNA polymerase but not DNA polymerase.

MeCCNU gives rise to both an alkylating moiety and a carbamoylating moiety (5). Its lethal effects may be due to cross-linking DNA since a resistant cell line repairs cross-links while a sensitive line does not (4).

The most effective combinations of drugs also have an effect on mouse coat color in our experiments, suggesting that the antitumor effect is not simply a general cytotoxicity, but that it may be specific for pigment-forming cells. DTIC has been shown to produce antigenic changes in other types of tumor cells (1). It has also been shown to increase cellular tyrosine hydroxylase activity in neuroblastoma cells in culture (3). This induction of the melanin-biosynthetic pathway also occurs in B16 melanoma cells when exposed to imidazole, and analog of the same metabolite as DTIC (13). If DTIC also induces tyrosinase in melanoma cells, it could make the cells more immunogenic and/or play some role in rendering them more susceptible to a subsequent exposure to the alkylating effects of MeCCNU. It should be noted, however, that we occasionally observe a change in coat color after high doses of MeCCNU alone. Since ionizing radiation and radiomimetic drugs produce premature aging and coat color changes, these observations may only be fortuitous.

The doubling time of HP in $CD2F_1$ mice was reported previously by us to be 2.0 days (8). Our strain of this tumor now grows more rapidly, with a doubling time of 1.1 days. With HP, the optimum spacing for DTIC and MeCCNU in the present experiments is 1 day. The scheduling effect observed could be related in some way to the tumor cell-doubling time.

The model system of sequential administration of single doses used in these experiments is a simple and effective way to search for therapeutic synergism. Initiation of therapy shortly after tumor cell injection appears to be the most sensitive means of revealing synergism, since minimally effective doses of the single agents can be used. Once enhancement has been revealed, administration of the drugs at later times and in higher doses shows that the appropriate schedule of DTIC and MeCCNU still produced an enhancing effect.

Observations that are true for one strain of murine melanoma but not for another indicate that general rules are difficult to formulate which respect to drugs, doses, and schedules even for a single tumor type, such as melanoma. These studies emphasize the necessity to individualize treatment plans in therapy to allow for individual responses of both the tumor and the host. *In vitro* assays which would enable the optimum drugs, doses, and schedules to be selected would be of great benefit, inasmuch as present methods of therapy are based mainly on historical data and observations of fortuitous responses to empirically selected treatment.

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