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Multi-chemothermoimmunotherapy for human colon adenocarcinoma in vitro

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Abstract Effective adjunctive therapies for colorectal carcinoma are clearly needed. We evaluated the cytotoxic responses in vitro of human colon carcinoma cell lines to combined modalities: 5-fluorouracil/leucovorin (5-FU/LV), carboplatin (CP), tumor necrosis factor (TNF) and hyperthermia (HTX). Cytotoxicity was evaluated in a cell proliferation assay using crystal violet staining. 5-FU/LV was administered 2–3 days before TNF and CP, followed 1 h later by HTX. These cell lines were relatively resistant to HTX alone (42°C for 2 h), but were heterogeneous in their responses to various doses of the other single agents. This heterogeneity was also evident for combined modalities: the HCT-15 cell line exhibited significant supra-additivity for selected doses of CP, TNF and 5-FU/LV, which was further enhanced by hyperthermia. In contrast, the HT-29 cell line did not demonstrate a strong pattern for supra-additivity, whereas the DLD-1 cell line had an intermediate response. Thus, our results suggest one approach to develop effective and dose-sparing multimodality therapeutic regimens for colon adenocarcinoma.

Key words Chemotherapy · TNF · Hyperthermia
Colon Adenocarcinoma · Multimodality

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Introduction

Colorectal carcinoma is an increasingly devastating disease, with over 138,000 new cases diagnosed annually in the US [2]. Despite locally effective surgical intervention for primary and known metastatic disease sites, the prognosis is dismayingly poor [2, 5, 6, 18]. Most chemotherapeutic regimens directed at residual disease have involved 5-fluorouracil (5-FU) combined with leucovorin (LV) which has in some cases resulted in improved response rates compared to 5-FU alone [7, 18, 21–23, 28]. A number of other agents have also been used in combination with 5-FU, including interferon-alpha [19, 24], tumor necrosis factor (TNF) [19] and platinum drugs [3, 9, 13, 15, 20, 29].

TNF has been evaluated clinically in colorectal carcinoma, including phase II trials [17, 25]. Systemic routes of administration have yielded disappointing results including toxicities evident in numerous other phase I/phase II trials [25, 27]. However, regional administration of TNF via hepatic arterial infusion to colorectal carcinoma patients with chemotherapy-resistant hepatic metastases [17] has resulted in encouraging response rates (5 of 14). Moreover, the tolerated doses using this route have been found to be sixfold higher than with systemic administration. Thus, current evidence from this trial and from others [14, 30] strongly suggests that TNF needs to be re-evaluated for regional rather than systemic administration. The possibility of using TNF in combination with 5-FU/LV in this setting should be considered.

We have previously reported that TNF, carboplatin (CP) and hyperthermia (HTX) interact strongly to generate supra-additive cytotoxicity against human colon carcinoma cell lines in vitro [11]. Only recently has 5-FU been shown to be a heat-interactive drug in vitro and in vivo [10, 16, 26]. Given the current clinical evaluation of combined 5-FU/LV and platinum, an established heat-interactive drug, in colorectal cancer trials [3, 9, 13, 15, 20, 29], we sought in this study to define cytotoxic interactions between 5-FU/LV, CP, TNF, and HTX against a panel of human colon tumor cell lines in vitro.

Materials and methods

Target cells and cell culture

The target cells were human colon adenocarcinoma cell lines DLD-1, HCT-15 and HT-29, with epithelial-like morphology and which display tumorigenicity in nude mice. These were purchased from the ATCC (Rockville, Md.). Their maintenance has been described previously [11].

Crystal violet cytotoxicity assays

Tumor cells (750–1000) were seeded in 180 µl medium per well of replicate 96-well plates on day + 1. On day 2, 20 µl LV (yielding a final concentration of 10 µ*M*) (Lederle Parenterals, Carolina, Puerto Rico) with or without three different doses of 5-FU (Lyphomed, Deerfield, Ill.) was added to each well, except for control wells. On day 4 or 5, 10 µl rHuTNF (Genentech, South San Francisco, Calif.) dissolved in medium was added at two different doses. Control wells received medium only. Immediately thereafter, 10 µl CP (Bristol Myers-Squibb, Evansville, Ind.) dissolved in sterile water was added at two different doses to designated wells. Additional controls included cells treated with TNF alone and those that received neither TNF nor CP. These manipulations were made as rapidly as possible and then the plates were incubated at 37°C for 1 h.

At that time, parallel sets of plates were removed, bagged in an atmosphere of 5% CO_2/air and then immersed for 2 h in water baths either at 37°C (control) or 42°C. At the end of the 2-h treatment, the plates were removed from the bags and incubated for 5 days at 37°C. The remaining viable cells were fixed and stained with crystal violet (Sigma). Bound dye was solubilized by treatment with methanol, and the optical density at 590 nm was determined in an automated plate reader. The raw optical density values were normalized to a 100% survival value for normothermic control cells. Percent cytotoxicity was calculated as the ratio of the optical densities from experimental vs control cultures.

Statistical methods

The data were first analyzed by the Response Surface Analysis [4] in the SAS statistical package (SAS Institute, Cary, N.C.). The percentage kill was transformed into a logistic response, i.e. for each percentage kill, p, the quantity Logit = ln $[p/(1\text{-}p)]$ was regressed against logarithms of the combination doses, except for the heat and platinum; for zero doses, a very small quantity was substituted before taking the logarithm. All the treatment modalities could be incorporated into a single response surface model.

Since dose-response relationships based on the logarithms of the doses are not appropriate to study the question of supra-additivity of the combination of modalities, a second approach was used [1]. We assumed that the differing modalities acted independently and that only additive cytotoxicity would occur under the null hypothesis, i.e. $H_0: 1 - P_m = (1 - P_1)(1 - P_2)(1 - P_3)\ldots$. Then, if the modalities did interact and result in supra-additivity, the following relation would hold:

$$1 - P_m < (1 - P_1)(1 - P_2)(1 - P_3)\ldots$$

where P_m is the percentage kill under the multimodality treatment and P_i ($i = 1, 2, 3, \ldots$) is the percentage kill by one drug used singly. The equation under the null hypothesis is equivalent to:

$$\log(1 - P_m) = \log(1 - P_1) + \log(1 - P_2) + \log(1 - P_3) + \ldots$$

Thus, the logarithm of the survival can be analyzed by the analysis of variance for an additive model. The mean square error from the replicate samples was used to calculate the t-values in testing the hypothesis of independence of the modalities in combination treatment.

Results

The results shown in Tables 1–3 for the individual cell lines reflect observed cytotoxicity and values calculated based on pure additivity. The response surface analysis shown in Tables 4–6 indicates the tendencies of the cytotoxic responses over the dose ranges for each modality and also whether additional responses are evident upon combination of modalities. The values in Tables 7 and 8 reflect the t-values from testing the null hypothesis for the independence of the modalities.

DLD-1 cells

Single treatments and dual combination with LV

CP, added to LV-containing cultures at doses of 2.5 and 5.0 µg/ml, demonstrated slight cytotoxicity (8.4%)

Table 1 Percent cytotoxicity of individual and multiple modalities against DLD-1 cell line. Data normalized to 0 for the normothermic, LV response (6.1% compared to untreated controls). Values are means: values in parentheses are the calculated percentage cytotoxicity for a purely additive response (*H*, *M*, *L* high, medium, and low doses, respectively; i.e. 312, 78, and 19.5 ng/ml 5-FU, and 270, and 10 ng/ml TNF)

Treatment	CP dose		
	0 µg/ml	2.5 µg/ml	5 µg/ml
37°C			
LV	0.0	− 2.4	8.4
5-FU-H	30.8	36.7 (29.1)	46.3 (36.6)
5-FU-M	− 6.1	− 0.7 (− 8.6)	6.8 (2.8)
5-FU-L	− 6.7	− 4.4 (− 9.3)	0.4 (2.3)
TNF-H	5.1	7.3 (2.8)	15.8 (13.1)
TNF-L	1.8	2.2 (− 0.6)	8.6 (10.0)
5-FU-H/TNF-H	48.4 (34.3)	58.3 (32.7)	67.0 (39.8)
5-FU-H/TNF-L	32.3 (32.0)	45.5 (30.4)	56.9 (37.8)
5-FU-M/TNF-H	10.9 (− 0.7)	10.4 (− 3.1)	25.6 (7.8)
5-FU-M/TNF-L	− 7.6 (− 4.2)	1.1 (− 6.7)	12.3 (4.6)
5FU-L/TNF-H	1.0 (− 1.3)	2.1 (− 3.7)	13.1 (7.2)
5-FU-L/TNF-L	− 3.4 (− 4.8)	− 4.1 (− 7.3)	5.6 (4.0)
42°C			
LV	0.5	8.1 (1.9)	15.1 (8.9)
5-FU-H	34.9 (31.1)	46.6 (29.5)	52.0 (36.9)
5-FU-M	8.1 (− 5.6)	20.0 (− 8.1)	20.0 (− 3.3)
5-FU-L	3.2 (− 6.2)	8.0 (− 8.8)	15.9 (2.8)
TNF-H	12.9 (5.6)	17.9 (3.3)	31.4 (13.5)
TNF-L	4.3 (2.3)	12.2 (− 0.1)	23.6 (10.5)
5-FU-H/TNF-H	56.6 (34.6)	65.7 (33.0)	71.0 (40.1)
5-FU-H/TNF-L	48.5 (32.3)	59.2 (30.7)	65.0 (38.1)
5-FU-M/TNF-H	20.5 (− 0.2)	29.5 (− 2.6)	42.7 (8.3)
5-FU-M/TNF-L	9.7 (− 3.7)	18.6 (− 6.2)	28.9 (5.1)
5-FU-L/TNF-H	22.0 (− 0.8)	21.9 (− 3.2)	31.3 (7.7)
5-FU-L/TNF-L	5.9 (− 4.3)	14.6 (− 6.8)	21.3 (4.5)

Table 2 Percent cytotoxicity of individual and multiple modalities against HCT-15 cell line. Data normalized to 0 for the normothermic, LV response (− 0.7% compared to untreated controls). Values are means: values in parentheses are the calculated percentage cytotoxicity for a purely additive response (*H*, *M*, *L* high, medium, and low doses, respectively; i.e. 78, 9.6, and 1.2 ng/ml 5-FU, and 30, and 1.1 ng/ml TNF)

	CP dose		
Treatment	0 μg/ml	2.5 μg/ml	5 μg/ml
37°C			
LV	0.0	0.6	27.5
5-FU-H	− 0.4	3.9 (0.2)	39.0 (27.2)
5-FU-M	5.2	10.6 (5.8)	30.7 (31.3)
5-FU-L	1.1	6.4 (1.7)	29.4 (28.3)
TNF-H	42.0	63.8 (42.3)	85.0 (58.0)
TNF-L	1.6	8.3 (2.2)	48.4 (28.7)
5-FU-H/TNF-H	55.9 (41.8)	68.7 (42.1)	90.2 (57.8)
5-FU-H/TNF-L	4.0 (1.2)	17.3 (1.8)	65.0 (28.1)
5-FU-M/TNF-H	53.5 (45.0)	73.4 (45.3)	87.8 (60.1)
5-FU-M/TNF-L	23.4 (6.7)	15.6 (7.3)	51.0 (32.2)
5FU-L/TNF-H	45.9 (42.6)	65.9 (43.0)	91.0 (58.4)
5-FU-L/TNF-L	15.1 (2.7)	18.0 (3.3)	50.4 (29.4)
42°C			
LV	2.4	14.0 (3.0)	62.1 (29.2)
5-FU-H	21.3 (2.0)	28.2 (2.6)	69.2 (28.9)
5-FU-M	10.1 (7.5)	41.2 (7.1)	76.6 (32.9)
5-FU-L	16.8 (3.5)	40.3 (4.1)	71.3 (30.0)
TNF-H	71.9 (43.4)	87.8 (43.7)	94.3 (59.0)
TNF-L	12.6 (4.0)	39.9 (4.5)	71.1 (30.4)
5-FU-H/TNF-H	78.3 (43.2)	91.0 (43.5)	94.9 (58.8)
5-FU-H/TNF-L	37.0 (3.6)	54.6 (4.2)	79.9 (29.8)
5-FU-M/TNF-H	83.0 (46.3)	93.0 (46.6)	96.6 (61.1)
5-FU-M/TNF-L	38.6 (8.9)	64.8 (9.5)	91.7 (33.8)
5-FU-L/TNF-H	82.3 (44.0)	94.7 (44.4)	96.6 (59.4)
5-FU-L/TNF-L	41.7 (5.0)	65.6 (5.6)	90.2 (31.1)

Table 3 Percent cytotoxicity of individual and multiple modalities against HT-29 cell line. Data normalized to 0 for the normothermic, LV response (− 0.7% compared to untreated controls). Values are means: values in parentheses are the calculated percentage cytotoxicity for a purely additive response (*H*, *M*, *L* high, medium, and low doses, respectively; i.e. 312, 39 and 5 ng/ml 5-FU, and 30, and 1.1 ng/ml TNF)

	CP dose		
Treatment	0 μg/ml	2.5 μg/ml	5 μg/ml
37°C			
LV	0.0	5.6	15.3
5-FU-H	51.8	55.8 (54.5)	58.2 (59.2)
5-FU-M	6.0	11.7 (11.3)	23.2 (20.4)
5-FU-L	3.6	6.3 (9.0)	16.8 (18.3)
TNF-H	24.7	32.9 (28.9)	54.0 (36.2)
TNF-L	3.6	4.1 (9.0)	15.7 (18.3)
5-FU-H/TNF-H	79.1 (63.7)	82.4 (65.7)	83.3 (69.3)
5-FU-H/TNF-L	52.9 (53.5)	55.2 (56.1)	62.3 (60.6)
5-FU-M/TNF-H	41.2 (29.2)	53.6 (32.5)	63.3 (40.0)
5-FU-M/TNF-L	13.2 (9.4)	14.1 (14.5)	29.8 (23.2)
5FU-L/TNF-H	30.6 (27.4)	38.0 (31.5)	56.5 (38.5)
5-FU-L/TNF-L	7.6 (7.1)	8.7 (12.3)	24.5 (21.3)
42°C			
LV	5.3	13.0 (10.6)	30.3 (19.8)
5-FU-H	62.1 (54.4)	64.5 (56.9)	70.0 (61.4)
5-FU-M	8.0 (11.0)	11.5 (16.0)	32.1 (24.6)
5-FU-L	3.8 (8.7)	8.2 (13.8)	28.5 (22.6)
TNF-H	40.9 (28.7)	52.7 (32.7)	67.9 (39.6)
TNF-L	9.4 (8.7)	15.0 (13.8)	39.9 (22.6)
5-FU-H/TNF-H	87.6 (65.6)	87.8 (71.0)	89.7 (70.9)
5-FU-H/TNF-L	68.2 (56.0)	65.9 (58.4)	73.6 (62.7)
5-FU-M/TNF-H	44.4 (33.0)	50.8 (36.1)	65.3 (43.2)
5-FU-M/TNF-L	13.7 (14.2)	14.2 (19.0)	39.6 (27.3)
5-FU-L/TNF-H	34.3 (31.2)	42.6 (35.1)	57.5 (41.8)
5-FU-L/TNF-L	9.9 (12.0)	11.9 (16.9)	33.4 (25.5)

only at the higher dose (Table 1). Likewise, when 5-FU was added to LV-containing cultures over a dose-range from 19.5 to 312 ng/ml, only the highest dose was markedly cytotoxic (30.8%, Table 1). DLD-1 cells were highly resistant to even the high dose level of TNF (270 ng/ml; 5.1% cytotoxicity; Table 1). By the response surface analysis, all single modalities (HTX, CP, TNF, 5FU) achieved significant responses ($P < 0.001$).

Multiple combinations

The analysis of variance of the log-linear model did not reveal significant multiple-modality interactions (no F-value ≥ 3.00) when evaluated over the full dose ranges (Table 4); only TNF combined with 5-FU resulted in marginal significance ($F = 1.41$; $p = 0.21$). However, at selected doses, DLD-1 cells demonstrated substantial supraadditivity (t values > 1.96) in their response to the chemotherapeutic agents and TNF (Tables 1 and 7). When the highest dose of TNF (270 ng/ml) was combined with high dose CP (5 μg/ml) and high-dose 5-FU (312 ng/ml) at normothermic or hyperthermic temperatures, the t-values of 2.33 and 2.51, respectively, indicated significant supra-additivity ($P < 0.05$; Table 7). Similarly, strong supra-additive responses were observed with high-dose TNF and 5-FU combined with low-dose CP (2.5 μg/ml) and HTX ($t = 2.29$) or low-dose TNF (10 ng/ml) combined with high doses of 5-FU and CP along with hyperthermia ($t = 1.98$).

HCT-15 cells

Single treatments and dual combination with LV

CP was cytotoxic at the higher dose (5 μg/ml; 27.5% cytotoxicity), but not at the lower dose (Table 2). HCT-15 cells were resistant to even the highest dose of 5-FU (78 ng/ml) examined (Table 2). However, they were the most sensitive of the three lines to TNF, with a dose of 30 ng/ml causing 42.0% cytotoxicity (Table 2). In the response surface analysis, all single modalities achieved significant responses ($P < 0.001$–0.003).

Table 4 Analysis of variance of log-linear model of DLD-1 cell response to multiple modalities

Source	Degrees of Freedom	Type I Sum of Squares	Mean Square	*F*-value[a]	*P* value
HTX	1	2.7728	2.7728	28.15	0.0001
CP	2	3.1796	1.5898	16.14	0.0001
HTX/CP	2	0.1036	0.0518	0.53	0.5918
TNF	2	3.1385	1.5692	15.93	0.0001
HTX/TNF	2	0.1156	0.0578	0.59	0.5570
CP/TNF	4	0.1628	0.0407	0.41	0.7990
HTX/CP/TNF	4	0.0119	0.0030	0.03	0.9982
5-FU	3	29.6865	9.8955	100.45	0.0001
HTX/5-FU	3	0.1468	0.0489	0.50	0.6850
CP/5-FU	6	0.4703	0.0784	0.80	0.5742
HTX/CP/5-FU	6	0.0554	0.0092	0.09	0.9969
TNF/5-FU	6	0.8361	0.1393	1.41	0.2102
HTX/TNF/5-FU	6	0.0547	0.0091	0.09	0.9970
CP/TNF/5-FU	12	0.0537	0.0045	0.05	1.0000
HTX/CP/TNF/5-FU	12	0.0376	0.0031	0.03	1.0000
Error	216	21.2792	0.0985		

[a] *F*-values $\geq$ 3.00 for multiple interactions would be considered to demonstrate interaction

Table 5 Analysis of variance of log-linear model of HCT-15 cell response to multiple modalities

Source	Degrees of Freedom	Type I Sum of Squares	Mean Square	*F*-Value[a]	*P* value
HTX	1	45.1510	45.1510	157.83	0.0001
CP	2	98.9347	49.4673	172.91	0.0001
HTX/CP	2	3.3736	1.6868	5.90*	0.0030
TNF	2	203.2017	101.6009	355.15	0.0001
HTX/TNF	2	2.5454	1.2727	4.45*	0.0124
CP/TNF	4	11.3393	2.8348	9.91*	0.0001
HTX/CP/TNF	4	4.5523	1.1381	3.98*	0.0036
5-FU	3	5.6168	1.8723	6.54	0.0003
HTX/5-FU	3	2.8175	0.9392	3.28*	0.0211
CP/5-FU	6	0.1467	0.0244	0.09	0.9976
HTX/CP/5-FU	6	0.8279	0.1380	0.48	0.8215
TNF/5-FU	6	0.5710	0.0952	0.33	0.9195
HTX/TNF/5-FU	6	0.3880	0.0647	0.23	0.9682
CP/TNF/5-FU	12	0.3509	0.0292	0.10	1.0000
HTX/CP/TNF/5-FU	12	0.4819	0.0401	0.14	0.9997
Error	344	98.4113	0.2861		

* *F*-values $\geq$ 3.00 for multiple interactions would be considered to demonstrate interaction

Multiple combinations

5-FU had little effect over the dose range used when combined with other modalities except for HTX. When high-dose 5FU (78 ng/ml) was combined with HTX, 21.3% cytotoxicity was observed, whereas a purely additive response would have given only 2.0% cytotoxicity (Table 2). This interaction was also reflected in a significant *F*-value (3.28; $P < 0.022$; Table 5). High-dose CP combined with high-dose TNF exerted the greatest cytotoxic effect of any dual combination (85.0%; Table 2), which could be enhanced by HTX (94.3% observed, 59.0% calculated; Table 2). Thermal enhancement was also observed for CP (27.5% to 62.1%) and for TNF (42.0% to 71.9%) alone compared with the normothermic responses to these agents (Table 2). This was also born out by response surface analysis, which demonstrated supra-additivity between CP and HTX ($P < 0.003$), TNF and HTX ($P < 0.013$), and in the triple combination ($P < 0.004$). As seen in lines 9, 11, 13, 15, 17, 19 and 21 of Table 8, 7 of 36 possible combinations of CP (three levels), TNF (three levels) and 5-FU (four levels) at 37°C exhibited supra-additivity ($t > 1.96$; $P < 0.05$). This proportion rose to 21 of 36 combinations with hyperthermia (lines 10, 12, 14, 16, 18, 20, 22–30, Table 8).

HT-29 cells

Single treatments and dual combination with LV

CP exhibited a dose-dependent cytotoxic effect on these cells under normothermic conditions (5.6%

Table 6 Analysis of variance of log-linear model of HT-29 cell response to multiple modalities

Source	Degrees of freedom	Type I sum of squares	Mean square	*F*-value[a]	*P* value
HTX	1	2.9088	2.9088	8.19	0.0046
CP	2	5.2990	2.6495	7.46	0.0007
HTX/CP	2	0.2575	0.12878	0.36	0.6962
TNF	2	24.1892	12.0946	34.07	0.0001
HTX/TNF	2	0.2712	0.1356	0.38	0.6830
CP/TNF	4	0.4015	0.1004	0.28	0.8890
HTX/CP/TNF	4	0.0262	0.0066	0.02	0.9993
5-FU	3	57.2646	19.0882	53.77	0.0001
HTX/5-FU	3	0.9223	0.3074	0.87	0.4595
CP/5-FU	6	0.4789	0.0798	0.22	0.9684
HTX/CP/5-FU	6	0.0711	0.0118	0.03	0.9998
TNF/5-FU	6	0.9994	0.1666	0.47	0.8307
HTX/TNF/5-FU	6	0.2140	0.0357	0.10	0.9963
CP/TNF/5-FU	12	0.1551	0.0129	0.04	1.0000
HTX/CP/TNF/5-FU	12	0.0486	0.0040	0.01	1.0000
Error	216	76.6798	0.3540		

[a] *F*-values ≥ 3.00 for multiple interactions would be considered to demonstrate interaction

Table 7 Supra-additive interactions of CP, TNF, 5-FU and HTX against the DLD-1 CELL line

		Percent Cytotoxicity Combined			
Modality	Dose	Single	Expected	Observed	*t*-value
CP	5 μg/ml	10.2	47.6	72.4	2.33*
TNF	270 ng/ml	6.7			
5-FU	312 ng/ml	37.4			
CP	2.5 μg/ml	3.3	44.1	71.2	2.29*
TNF	270 ng/ml	6.7			
5FU	312 ng/ml	37.4			
HTX		1.1			
CP	5 μg/ml	10.2	46.1	71.1	1.98*
TNF	10 ng/ml	3.0			
5-FU	312 ng/ml	37.4			
HTX		1.1			
CP	5 μg/ml	10.2	48.1	76.4	2.51*
TNF	270 ng/ml	6.7			
5-FU	312 ng/ml	37.4			
HTX		1.1			

* $P < 0.05$ ($t = 1.96$)

cytotoxicity at 2.5 μg/ml and 15.3% at 5 μg/ml; Table 3). 5-FU at the highest dose (312 ng/ml) caused pronounced cytotoxicity (51.8%; Table 3) in the presence of LV. The response to TNF was intermediate for the three cell lines, with a dose of 30 ng/ml causing 24.7% cytotoxicity (Table 3). In the response surface analysis, all single agents induced significant responses ($P < 0.0001$ for 5-FU and for TNF; $P < 0.0007$ for CP; $P < 0.005$ for HTX).

Multiple combinations

Under normothermic conditions, while all the individual agents were effective (Table 3), there was no strong pattern of supra-additivity with combinations; no *F*-value exceeded 0.87 (Table 6), and no *t*-value exceeded 1.11 (data not shown).

Discussion

The current studies were motivated by the clinical reality of generally poor response rates and a lack of durable responses to 5-FU regimens currently employed as adjunctive therapies in colorectal adenocarcinoma. If combinations of cytotoxic modalities with both apparently distinct mechanisms of action and in vivo toxicities could achieve similar antitumor effects at reduced doses or greater antitumor effects with high (near MTD) doses [31], perhaps treatments could be repeated, tumor resistance reduced, response rates improved and/or responses made more durable. Hence, the motivation for the current studies.

The three human colon tumor cell lines used exhibited heterogeneity in their responses to the individual modalities (Tables 1–3) as well as in their responses to the combined modalities (Tables 4–8). It is of interest that the responses to 5-FU and TNF appeared to be reciprocal; for example, HCT-15 cells were sensitive to TNF but resistant to 5-FU; DLD-1 cells were sensitive to 5-FU but highly resistant to TNF; HT-29 cells were sensitive to both. These findings suggest that the resistance mechanisms are likely distinct. We did not determine whether the cytotoxic responses to TNF and/or 5-FU were apoptotic, necrotic or mixed. If both agents induce the same pathway(s), future studies may be directed to ascertaining the molecular basis for their interaction.

Furthermore, the sensitivity to 5-FU correlated inversely with the response to HTX. For example, the

Table 8 Supra-additive interactions of CP, TNF, 5-FU and HTX against the HCT-15 cell line. CP doses are in μg/ml, and TNF and 5-FU doses are in ng/ml

Modalit/dose	*t*-value	Percent cytotoxicity (geometric mean)	
		Expected	Observed
CP (2.5)	—	—	0.6
CP (5.0)	—	—	32.5
TNF (1.1)	—	—	1.7
TNF (30)	—	—	41.7
5-FU (1.2)	—	—	1.1
5-FU (9.6)	—	—	5.4
5-FU (78)	—	—	0.0
HTX	—	—	2.4
CP(2.5); TNF(30); 5-FU(78)	2.61**	41.8	80.3
CP (2.5); TNF (30); 5-FU (78); HTX	3.95**	43.2	91.4
CP(2.5); TNF(30); 5-FU(9.6)	2.47*	45.0	80.2
CP (2.5); TNF (30); 5-FU (9.6); HTX	4.51**	46.3	93.8
CP (2.5); TNF(30); 5-FU(1.2)	2.29*	42.7	77.8
CP (2.5); TNF (30); 5-FU (1.2); HTX	4.87**	44.0	94.5
CP (5); TNF (30)	4.11**	60.7	90.2
CP (5);TNF (30); HTX	4.62**	61.6	94.3
CP(5); TNF (30); 5-FU (78)	4.05**	60.5	92.6
CP (5); TNF (30); 5-FU (78); HTX	4.17**	61.5	94.8
CP (5); TNF (30); 5-FU (9.6)	3.37**	62.7	90.8
CP (5); TNF (30); 5-FU (9.6); HTX	4.67**	63.6	96.1
CP (5); TNF (30); 5-FU (1.2)	3.79**	61.1	91.9
CP (5); TNF (30); 5-FU (1.2); HTX	4.84**	62.0	96.2
TNF (30); HTX	2.18*	43.1	72.8
TNF (30); 5-FU (78); HTX	2.38*	42.9	78.7
TNF (30); 5-FU (9.6); HTX	2.74**	46.0	82.7
TNF (30); 5-FU(1.2); HTX	2.79**	43.7	82.3
CP (2.5); TNF (30); HTX	4.04**	43.4	89.3
CP (2.5); TNF (1.1); 5-FU (9.6); HTX	2.09*	9.5	66.7
CP (2.5); TNF (1.1); 5-FU (1.2); HTX	2.12*	5.6	65.7
CP (5); 5-FU (78); HTX	2.10*	33.9	72.3
CP (5); 5-FU (9.6); HTX	2.60**	37.6	78.8
CP (5); 5-FU (1.2); HTX	2.09*	34.9	72.6
CP (5); TNF (1.1); HTX	2.84*	35.2	80.0
CP (5); TNF (1.1); 5-FU (78); HTX	3.04**	34.9	84.8
CP (5); TNF (1.1); 5-FU (9.6); HTX	4.12**	38.6	91.4
CP (5); TNF (1.1); 5-FU (1.2); HTX	4.23**	35.9	91.5

*$P < 0.05$ ($t = 1.96$) **$P < 0.01$ ($t = 2.58$)

5-FU-sensitive HT-29 cells did not show heat interaction (nor with other modalities); DLD-1 cells which were sensitive only to high-dose 5-FU also showed thermal sensitization to 5-FU-containing combinations; and HCT-15 cells demonstrated resistance to 5-FU, but marked thermal enhancement, particularly with combinations including lower doses of 5-FU and CP. HTX could modulate the cellular uptake and/or metabolism of these drugs. Collectively, these findings indicate a strong role for HTX in overcoming tumor cell resistance or enhancing the response to chemotherapeutic or biological modalities or their combinations.

The current studies use modalities in a clinically applicable fashion. Whole-body hyperthermia of colorectal carcinoma patients can be conducted at up to 41.8°C (J.M.C. Bull, personal communication). The doses of 5-FU and CP are within those levels reported from pharmacokinetics studies of these drugs [8, 12]. Hepatic arterial infusion of TNF [17] allows doses as high as 150 μg/m^2 to be administered for two weekly cycles of five doses per week. However, the pharmacokinetics of the infused TNF have not been established. It is not known whether the liver captures or inactivates the TNF, and certainly no direct measurement of intrahepatic TNF levels has been reported. Assuming a patient surface area of 1.5 m^2 and ~ 80% capture by the liver in a hepatic volume of distribution of 1 l, a peak intrahepatic level of 180 ng/ml could be achieved daily for up to 10 days, which is within the effective ranges for interactions demonstrated in our current studies. However, this simple model ignores the important indirect modes of antitumor action of TNF in vivo, which include modulation of blood flow and immune effector mechanisms. Therefore, the current in vitro studies can only be considered an initial step in developing a protocol based on the combination of these modalities. Future studies to evaluate such a protocol for its in vivo efficacy and toxicity would appear to be warranted.

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References

1. Berenbaum MC (1985) The effect of a combination of agents: the general solution. J Theor Biol 114: 413–431
2. Wingo, PA, Tong T, Bolden S (1995) CA – a cancer journal for clinicians. Cancer statistics 45: 8–30
3. Cantrell JE Jr, Hart RD, Taylor RF, Harvey JH Jr (1987) Pilot trial of prolonged continuous infusion 5-fluorouracil and weekly cisplatin in advanced colorectal cancer. Cancer Treat Rep 71: 615–618
4. Carter WH, Jones DE, Carchman RA (1985) Application of response surface methods for evaluating the interactions of soman, atropine and pralidioxime chloride. Fundam and Appl Toxicol 5: s232–s241

5. Cohen AM, Shank G, Friedman MA (1989) Colorectal cancer. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds) Cancer Principle and practice of oncology, JB Lippincott, Philadelphia, pp 895–952
6. Devita, VT Jr (1989) Principles of chemotherapy. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds) Cancer principle and practice of oncology, JB Lippincott, Philadelphia, pp 276–300
7. Erlichman C, Fine S, Wong A, Elhakim T (1988) A randomized trial of fluorouracil and folinic acid in patients with metastatic colorectal carcinoma. J Clin Oncol 6: 469–477
8. Harland SJ, Newell DR, Siddik ZH, Chadwick R, Calvert AH, Harrap KR (1984) Pharmacokinetics of cis-diammine-1,1-cyclobutane dicarboxylate platinum (II) in patients with normal and impaired renal function. Cancer Res 44: 1693–1697
9. Kemeny N, Israel K, Niedzwiecki D, Chapman D, Botet J, Minsky B, Winciguena V, Rosenbluth R, Bosselli B, Cochran C, Sheehan K (1990) Randomized study of continuous infusion fluorouracil versus fluorouracil plus cisplatin in patients with metastatic colorectal cancer. J Clin Oncol 8: 313–318.
10. Kido Y, Kuwano H, Maehara Y, Mori M, Matsuoka H, Sugimachi K (1991) Increased cytotoxicity of low-dose, long-duration exposure to 5-fluorouracil of V-79 cells with hyperthermia. Cancer Chemother Pharmacol 28: 251–254
11. Klostergaard J, Leroux E, Siddik ZH, Khodadadian M, Tomasovic SP (1992) Enhanced sensitivity of human colon tumor cell lines in vitro in response to thermochemoimmunotherapy. Cancer Res 52: 5271–5277
12. Lange J, Zanker KS, Siewert JR, Blumel G, Eisler K, Kolb E (1984) The effect of whole body hyperthermia on 5-fluorouracil pharmacokinetics in vivo and clonogenicity of mammalian colon cancer cells. Anticancer Res 4: 27–32
13. Levi F, Misset J-L, Brienza S, Adam R, Metzger G, Itzakki M, Caussanel J-P, Kunstlinger F, Lecouturier S, Descorps-Declere A, Jasmin C, Bismuth H, Reinberg A (1992) A chronopharmacologic Phase II clinical trial with 5-fluorouracil, folinic acid, and oxaliplatin using ambulatory multichannel programmable pump Cancer 69: 893–900
14. Lienard D, Ewalenko P, Delmotte I-J, Renard N, Lejeune FJ (1992) High-dose recombinant tumor necrosis factor alpha in combination with interferon gamma and melphalan in isolation perfusion of the limbs for melanoma and sarcoma. J Clin Oncol 10: 52–60
15. Loehrer PJ, Sr, Einhorn IH, Williams SD, Hui SL, Estes NC, Pennington K (1985) Cisplatin plus 5-fu for the treatment of adenocarcinoma of the colon. Cancer Treat Rep 69: 1359–1363
16. Maehara Y, Sakaguchi Y, Takahashi I, Yohisda M, Kusumoto H, Masuda H, Sugimachi K (1992) 5-fluorouracil's cytotoxicity is enhanced both in vitro and in vivo by combinant treatment with hyperthermia and dipyridamole. Cancer Chemother Pharmacol 29: 257–260
17. Mavligit GM, Zukiwski AA, Charnsangavej C, Carrasco CH, Wallace S, Gutterman JU (1992) Regional biologic therapy: hepatic arterial infusion of recombinant human tumor necrosis factor in patients with liver metastases Cancer 69: 557–561
18. Moertel CW, Fleming TR, Macdonald JS, Haller DG, Laurie JA (1990) Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N Engl J Med 322: 352–358
19. Naomoto Y, Muro M, Kataoka K, Fuchimoto S, Kurimoto M, Orita K (1991) Interferon-alpha (IFN-alpha), low-dose tumor necrosis factor-alpha (TNF-alpha) and 5FU in combination for advanced carcinoma: new rational treatment based on mechanism of actions. Proc Am Soc Clin Oncol PA1061
20. Palmeri S, Trave F, Russello O, Rustum YM (1989) The role of drug sequence in therapeutic selectivity of the combination of 5-fluorouracil and cisplatin. Selective Cancer Ther 5: 169–177.
21. Petrelli N, Herrera L, Rustum Y, Burke P, Creaven P, Stule J, Emrich LJ, Mittelman A 1987 A prospective randomized trial of 5-fluorouracil versus 5-fluorouracil and high-dose leucovorin versus 5-fluorouracil and methotrexate in previously untreated patients with advanced colorectal carcinoma. J Clin Oncol 5: 1559–1568
22. Petrelli N, Douglass HO Jr, Herrera L (1989) The modulation of fluorouracil with leucovorin in metastatic colorectal carcinoma: a prospective randomized phase III trial. J Clin Oncol 7: 1419–1428
23. Poon MA, O'Connell MJ, Moertel CG, Wiland HS, Gullinan SA, Everson LK, Krook JE, Mailliard JA, Laurie JA, Tschetter LK, Wiesnfeld M (1989) Biochemical modulation of fluorouracil: Evidence of significant improvement of survival and quality of life in patients with advanced colorectal carcinoma. J Clin Oncol 7: 1407–1416
24. Punt CJA, DE Mulder PHM, Burghouts JHM, Wagener DJH (1992) Alpha-interferon in combination with 5-fluorouracil and leucovorin in metastatic colorectal cancer: a Phase I study. Cancer Chemother Pharmacol 29: 326–328
25. Schaadt M, Pereundschuh MG, Lorscheidt G, Peters KM, Steinmetz T, Diehl V (1990) Phase II study of recombinant human tumor necrosis factor in colorectal carcinoma. J Biol Resp Mod 9: 247–250
26. Shiiki S, Fuchimoto S, Iwagaki H, Akazai Y, Matsubara N, Watanabe T, Orita K (1991) Comparative antitumor activity of 5-fluorouracil and its pro drugs in combination with hyperthermia in vitro. Acta Med Okagama 45: 339–345
27. Taguchi T (1986) A phase I study of recombinant human tumor necrosis factor. Jpn J Cancer Chemother. 13: 3491–3498
28. Valone FH, Friedman MA, Wittlinger PS, Drakes T, Eisenberg PD, Malec M, Hannigan JF, Brown BW, Jr (1989) Treatment of patients with advanced colorectal carcinomas with fluorouracil alone, high-dose leucovorin plus fluorouracil or sequential methotrexate, fluorouracil, leucovorin: a randomized trial of the Northern California Oncology Group. J Clin Oncol 7: 1427–1436
29. Vokes EE, Moormeier JA, Ratain MJ, Egorin MJ, Haraf DJ, Mick R, Weichselbaum RR (1992) 5-fluorouracil, leucovorin, hydroxyurea, and escalating doses of continuous-infusion cisplatin with concomitant radiotherapy: a clinical and pharmacologic study. Cancer Chemother Pharmcol 29: 178–184
30. Wakabayashi T, Yoshida J, Mizuno M, Sugita K, Yoshida T (1991) Effectiveness of tumor necrosis factor for malignant glioma: evaluation of its antitumor effects by CT and/or MRI. Biotherapy 5: 478–482.
31. Weitman SD, Kamen BA (1994) Combination chemotherapy. A case of the New Math? When an addition is a subtraction. Cancer Res Ther Control 4: 1–2