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Mechanisms of resistance to 6-thioguanine in a murine pancreatic tumor*

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Summary. PANC02 is a unique experimental animal tumor that fails to respond significantly to any known clinically active antitumor agent. In this regard, the murine ductal adenocarcinoma resembles its human counterpart. To study the mechanism for its intrinsic resistance to 6-thioguanine (TG), we compared the metabolism of the drug in PANC02 and a reference, TG-sensitive adenocarcinoma, CA-755. In comparison with CA-755, PANC02 cells were approximately 6 times less sensitive to TG and CHO cells were 80 times less sensitive in tissue culture. Nevertheless, the incorporation of TG into the DNA of these three cell lines was approximately equal at the lowest concentrations capable of reducing cloning efficiency by 50%, i.e., 3.0–3.8 pmol (dthioGMP)/nmol (dGMP). In mice bearing bilateral implants of CA-755 and PANC02, only CA-755 responded to TG treatment. At various doses used on various schedules, the incorporation of TG into CA-755 DNA readily achieved that observed to be cytotoxic to the cells in vitro, whereas the incorporation into the DNA of PANC02 tumor cells did not. Although the biochemical basis for the poor incorporation of TG into the DNA of PANC02 in vivo is not known, this factor appears to explain the refractoriness of PANC02 as compared with CA-755 to this antitumor antimetabolite.

Introduction

PANC02 is a ductal-cell adenocarcinoma of the pancreas that developed in a male C57/BL/6 mouse following treatment with 3-methylcholanthrene [2]. It is resistant to drugs representing every class of known, clinically active anticancer agent. The human counterpart of this and several other human solid tumors are also intrinsically resistant to anticancer drugs; therefore, it is important that the mechanisms of drug resistance in this unique experimental animal model be studied. Because of our previous experience in studying the mechanism underlying the antitumor activity of the antimetabolite 6-thioguanine (TG) [8], we performed a series of in vivo and in vitro experiments to ascertain the basis of the resistance of PANC02 to this antimetabolite. In the original report by Corbett et al. [2], TG and nine other antimetabolites failed to demonstrate activity against PANC02 at their maximal tolerated doses. For purposes of comparison, we used a mammary adenocarcinoma (CA-755) that also grows in C57/BL mice as a TG-sensitive solid tumor. The results suggest that one basis for the failure of PANC02 as compared with CA-755 to respond in vivo to TG is biochemical, namely, a reduction in TG metabolism to analog nucleotides and in its incorporation into nucleic acids, particularly DNA [11].

Materials and methods

Animal experiments: PANC02 and CA-755 tumors (obtained from Dr. J. Mayo, Frederick Cancer Research Facility, Frederick, Md.) were maintained by s.c. transplantation into the flank regions of male C57/BL/6 mice. Animals were treated with TG when the tumors had reached an advanced stage, i.e., ≥200 mg on days 9–12 following implantation. Tumor growth was determined by caliper measurement in two dimensions, and tumor size was estimated by assuming that the tumor was a prolate spheroid exhibiting a density equal to that of water [2, 10]. For determinations of TG metabolism in PANC02 and CA-755 tumors implanted on opposite flank regions, tumors were excised and rapidly homogenized in cold 0.5 *N* perchloric acid. After centrifugation, the cold, acid-soluble material was neutralized with 10 *N* KOH.

Incorporation of TG into RNA was determined in the acid-insoluble material after alkaline hydrolysis using high-performance liquid chromatography (HPLC) with fluorescent detection of the TG-oxidation product [6, 15]. Incorporation of TG into DNA was determined as dthioGMP after P1 nuclease digestion of DNA that had been isolated as described

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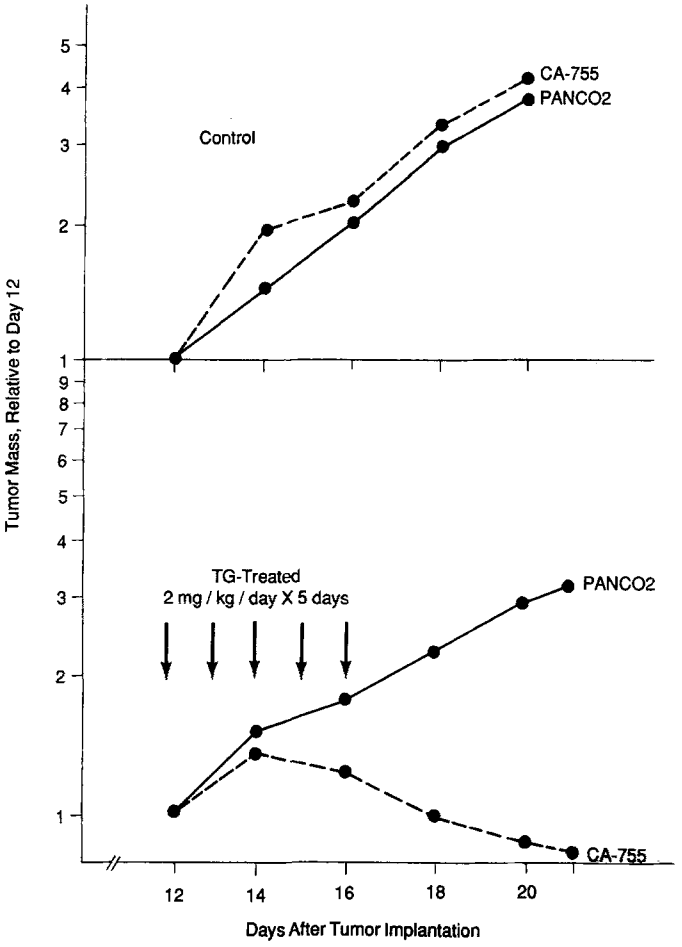

Fig. 1. Chemotherapeutic response of CA-755 and PANC02 to TG. The tumors were bilaterally implanted s.c. in the flank regions of C57BL/6 male mice. Drug treatment (2 mg/kg daily ×5) was begun on day 12 following tumor implantation. Tumor mass was estimated by caliper measurement [10]

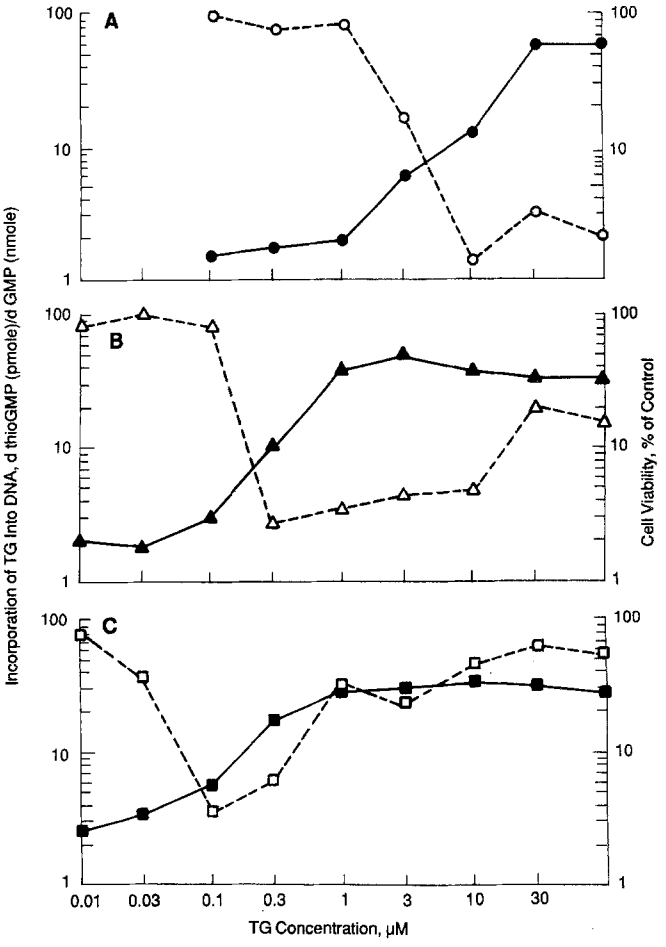

Fig. 2 A–C. Cytotoxicity (*open symbols*) and incorporation of TG into DNA (*closed symbols*) in cultured cells. **A** CHO, **B** PANC02, or **C** CA-755 cells were treated during exponential growth for 24 h at the indicated concentrations of TG. Incorporation of TG into DNA was determined immediately after drug treatment. Cell viability was determined 7 days there after according to colony formation. The ED_{50} value (μM) and TG incorporation into DNA at the ED_{50} (pmol dthioGMP/nmol dGMP) observed for the 3 cell lines were as follows: CHO, 1.5 and 3.0; PANC02, 0.12 and 3.8; and CA-755, 0.019 and 3.1. The basis for the reduced cytotoxicity observed at higher doses of TG is unknown, i.e., reflects self-limiting toxicity [1]

by Davis et al. [3]. DNA samples were denatured by placement in boiling water for 5 min followed by rapid chilling on ice for an additional 5 min. P1 nuclease (0.75 IU/100 μg DNA) and 0.1 vol. barbital buffer (300 mM barbital, 300 mM acetate, and 2 mM $ZnCl_2$) were then added. The samples were adjusted to pH 5.3 with 4 N HCl and incubated at 53° C for 1 h. After digestion, samples were adjusted to alkaline pH with 10 *N* KOH and passed through Ultrafree-MC membrane filters (Millipore Co., Bedford, MA) to remove the P1 nuclease. The nucleotides were separated using a Whatman SAX column as previously described [9]. Deoxyribonucleotide metabolites of TG were measured following the removal of ribonucleotides using periodate oxidation and base cleavage as described by Neu and Heppel [12].

Tissue-culture experiments. The culture line of PANC02 [16] was generously provided by Dr. L. J. Wilkoff of the Southern Research Institute (Birmingham, Ala.). The CA-755 cell line was established according to the method described by Freshney [5]. Freshly excised CA-755 tissue was chopped into fine pieces and passed through stainless-steel sieves to obtain a single-cell suspension. Initially, the suspension of single cells was cultured in Dulbecco's modified Eagle's medium (high-glucose) supplemented with 15% fetal bovine serum (heat-inactivated at 56° C for 30 min), 4 mM L-glutamine, 0.1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acid, 1×MEM essential amino acid, 0.1 mM oxalacetic acid, 8 μg crystalline insulin/ml and 10% NCTC 109 medium (Gibco BRL Life Technolgies, Inc., Grand Island, N. Y.). All three cell lines (CHO, PANC02, and CA-755) were maintained in McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 IU penicillin/ml, and 50 μg streptomycin/ml (Gibco BRL Life Technologies, Inc.) [4]. The response to TG in the cultured cells was determined by their exposure to various concentrations of TG for 24 h during exponential growth and subsequent determination of their cloning ability after 7–8 days as previously described [4]. The metabolism of TG and its incorporation into RNA and DNA were determined as described above.

Results and discussion

PANC02 and CA-755 grew at similar rates following their implantation on opposite flank regions of C57/BL/6 mice (Fig. 1). In mice treated with TG (2 mg/kg daily ×5, i.p.), CA-755 regressed, whereas PANC02 grew at essentially the rate observed in untreated controls. Thus, the sensitivity of CA-755 and the resistance of PANC02 to TG were confirmed in this model. In tissue culture, PANC02 cells were also more resistant to TG (about 6 times) than was CA-755 (Fig. 2). CHO cells were even more resistant to

Table 1. TG metabolism and ribonucleotide pools in CA-755 and PANC02 cells obtained from mice following treatment with TG at 2 mg/kg daily ×5[a]

Tumor	Mice (*n*)	Ribonucleotide pools (nmol/100 mg protein)					TG (pmol dthioGMP/nmol dGMP)	
		ATP+ADP	GTP+GDP	UDP+UTP	CTP	thioGMP	RNA	DNA
CA-755:								
Control	4	551 ±126	106 ±22	120 ±29	34 ±10	–	–	–
Days 3 + 5	8	705 ± 69	118 ±10	140 ±18	56 ± 7	8.0 ±1.2	1.9 ±0.3	2.7 ±0.5
PANC02:								
Control	4	738 ± 74	108 ±15	150 ±26	43 ±10	–	–	–
Days 3 + 5	8	729 ± 89	93 ±13	120 ±17	32 ± 6	5.1 ±1.0	0.89 ±0.19	0.60 ±0.09

[a] Groups of 4 animals each were killed at 2 h following TG treatment on days 3 and 5 as illustrated in Fig. 1. Tumors were rapidly excised and extracted with 0.5 *N* cold perchloric acid using a Brinkman Polytron homogenizer. Endogenous nucleotides and TG incorporation into RNA and DNA were determined as previously described [6, 15]. The results obtained for days 3 and 5 were quantitatively similar; consequently, the results were combined. Data represent mean values ± SE
ADP, Adenosine diphosphate; ATP, adenosine triphosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; CTP, cytosine triphosphate; GMP, guanosine monophosphate

Table 2. Incorporation of TG into DNA of PANC02 and CA-755 tumors in vivo[a]

Tumor	TG dose	TG incorporation (pmol dthioGMP/nmol dGMP)						
		+ 1 h	+2 h	+ 4 h	+ 8 h	+ 24 h	+ 72 h	+ 144 h
PANC02	2 mg/kg q4 h ×7	0.52 ±0.08	0.40 ±0.01	0.42 ±0.12	0.32 ±0.08	0.46 ±0.05	–	–
CA-755		3.4 ±0.9	3.5 ±0.5	3.4 ±1.2	3.2 ±1.0	5.7 ±0.6	–	–
PANC02	5 mg/kg q6 h ×4	–	1.9 ±1.1	–	–	–	–	–
CA-755		–	7.9 ±3.8	–	–	–	–	–
PANC02	10 mg/kg q24 h ×3	–	1.8 ±0.4	–	–	–	–	–
CA-755		–	15.0 ±1.8	–	–	–	–	–
PANC02	10 mg/kg q24 h ×2	–	–	–	–	0.74 ±0.29	0.50 ±0.09	0.12 ±0.02
CA-755		–	–	–	–	6.4 ±3.8	2.4 ±0.5	0.69 ±0.04

[a] Mice bearing bilateral implants of CA-755 and PANC02 were treated i.p. with various regimens of TG as shown. Animals were killed at different times after the last dose of drug, and the incorporation of TG into DNA was determined as described in Materials and methods. Data represent average values ± SE ($n = 5$)

TG as compared with PANC02 or CA-755 cells, the lowest concentrations of TG required to reduce cloning efficiency by 50% (ED_{50}) being 1.5, 0.12, and 0.019 μM, respectively. In spite of this 80-fold difference in sensitivity to TG in these 3 cell lines, the incorporation of TG into DNA at the ED_{50} concentrations was essentially the same, i.e., 3.0–3.8 pmol dthioGMP/nmol dGMP. The mechanism underlying the paradoxical reversal of cytotoxicity seen in the cultured cells (i.e., reduced toxicity at higher TG concentrations) is not known; however, in these experiments, the drug did not appear to reverse its own incorporation into DNA at higher drug concentrations as had previously been noted in CHO cells [1].

Measurement of TG metabolites in CA-755 and PANC02 tumors removed from mice bearing bilateral implants indicated that TG was distributed to both tumors to approximately the same extent (Table 1). Specifically, the thioGMP content and TG incorporation into RNA in the CA-755 tumor were only about 2-fold those observed in the PANC02 tumor. However, the incorporation of TG into DNA was 4.5 times lower in PANC02 than in CA-755 tumors (0.60 vs 2.7 pmol dthioGMP/nmol dGMP; Table 1). It may be relevant to note that this level of incorporation into CA-755 DNA approximates that associated with cytotoxicity in vitro. In contrast to the different patterns of TG metabolism, the endogenous nucleotide pools, RNA, protein, and DNA content of PANC02 and CA-755 tumors were remarkably similar (Table 1, data not shown).

To determine whether the availability of TG to the PANC02 tumor limited its incorporation into the DNA, several dose regimens were employed (Table 2). Following the administration of 2 mg/kg every 4 h for six doses, incorporation into PANC02 DNA was about 0.3–0.5 pmol dthioGMP/nmol dGMP during a 24-h interval following the last dose; in contrast, the level incorporated into CA-755 DNA was 3.2–5.7 pmol/nmol. In each case, persistence of TG in the DNA over this 24-h interval is apparent, i.e., if the TG is removed from the DNA this must occur at the time of insertion rather than at a subsequent time as has been inferred from previous reports [7, 13]. Even when larger doses of TG were given (i.e., 5 mg/kg g 6 h ×4 or 10 mg/kg g 24 h ×3), the incorporation of TG into PANC02 DNA did not achieve the level associated with cytotoxicity in cultured cells, namely 1.9 (Table 2) vs approx. 3 pmol dthioGmP/nmol dGMP (Fig. 2). The dose regimens shown in Table 2 were lethal to some of the

Table 3. Metabolism of TG in three tumor cell lines in tissue culture[a]

Cell	Incubation period (h)	Nucleotides (pmol/mg protein)					
		thioGMP	thioGDP	thioGTP	dthioGMP	dthioGDP	dthioGTP
CHO	1	1,705	24	37	135	<1	2
CHO	4	3,309	98	64	951	48	33
PANC02	1	603	188	862	81	4	11
PANC02	4	307	102	106	192	6	14
CA-755	1	125	83	90	3	<1	1
CA-755	4	151	92	82	29	<1	16

[a] Cells (5×10^7) were incubated with 10 μM TG during exponential growth for the periods shown and were then extracted with 0.5 *N* cold perchloric acid. TG metabolites in the acid-soluble, neutralized extracts were measured by HPLC using fluorescent detection as described in Materials and methods. Chemically synthesized dthioGMP and dthioGTP were used as standards for assignment of retention times thioGMP, thioGDP, thioGTP, dthioGMP, dthioGDP, dthioGTP

animals; however, during the interval examined, TG failed to reduce significantly the growth rate of PANC02, whereas that of CA-755 was markedly impaired (data not shown), similar to the result illustrated in Fig. 1. The rate of disappearance ($t_{1/2}$, ~36 h) of TG from the DNA of CA-755 or PANC02 tumors was similar following two doses of 10 mg/kg daily (last column, Table 2). However, it cannot be determined whether this apparent removal was attributable to biochemical mechanisms or to new DNA synthesis, since one tumor (CA-755) regressed during this interval whereas the other (PANC02) did not.

As discussed above, incorporation of TG into the DNA of PANC02 cells in vivo did not attain the level associated with toxicity to the cells in vitro, even when lethal doses of TG were used (Table 2, Fig. 2). A possible explanation for the refractoriness of CHO and PANC02 cells to TG as compared with CA-755 cells (Fig. 2) might be that the more resistant cells are more susceptible to inhibition of DNA synthesis due to TG treatment; that is, the drug may prevent its own incorporation into the DNA, thereby sparing the cells from TG-induced cytotoxicity [1, 17]. The mechanism whereby TG retards cell-cycle progression and inhibits DNA synthesis is not known. As shown in Table 3, TG nucleotide pools within CHO and PANC02 cells were actually higher than those in CA-755 cells following exposure to 10 μM TG. In fact, TG nucleotides did not appear to accumulate in CA-755 cells, perhaps due to the ready incorporation of TG into the RNA and DNA of this cell line. This experiment suggests that intracellular sequestration of TG nucleotides may contribute to the lower incorporation of TG into the DNA of PANC02 and CHO cells.

In summary, the intrinsic resistance of the PANC02 tumor as compared with the CA-755 tumor to TG appears to have a biochemical basis, namely, a reduced ability to incorporate the drug into DNA. This mechanism together with the finding that drug distribution within PANC02 tumors is more heterogeneous than that in CA-755 cells [14], provides a plausible explanation for the refractoriness of this interesting experimental tumor to TG. Another possibility that should be considered would be that the incorporation of TG into the DNA of CA-755 tumors is excessively high as compared with that in normal cells and that the incorporation into PANC02 tumors is more "normal-like".

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