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Research paper

Thymidylate synthase as a molecular target for drug discovery using the National Cancer Institute's Anticancer Drug Screen

Allyson L Parr,[1] Timothy G Myers,[2,3] Susan L Holbeck,[2,3] Yenlin J Loh[1] and Carmen J Allegra[1]

[1]Medicine Branch and [2]Information Technology Branch, Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, Bethesda, MD 20889, USA. [3]Present address: Large Scale Proteomics Corp, Rockville, MD 20950, USA.

Thymidylate synthase (TS) is a critical cellular target for cancer chemotherapeutics, particularly the fluoropyrimidine and antifolate classes of antineoplastic agents. One of the primary mechanisms of clinical insensitivity to these agents is through the overexpression of the target enzyme, TS. Thus, there is a need for the development of agents which selectively target TS-overexpressing malignant cells. To this end, we conducted a search for agents which potentially selectively target TS-overexpressing cells using two separate algorithms for identifying such compounds in the NCI Drug Repository by comparing cytotoxicity profiles of 30 000 compounds with the TS expression levels measured by Western blot analysis in 53 cell lines. Using the traditional COMPARE analysis we were unable to identify compounds which maintain a selective ability to kill high TS-expressing cells in a subsequent four cell line validation assay. A new algorithm, termed COMPARE Effect Clusters analysis, enabled the identification of a particular drug cluster which contained compounds that maintained a selective ability to kill TS-overexpressing cell lines in the validation assay. While the identified compounds were selectively cytotoxic to TS-overexpressing cells, we found that they were not specifically targeting TS as a mechanism of action. Apparently, the overexpression of TS was providing a marker for sensitivity. This identified class of compounds which appears to be selectively cytotoxic against cells which overexpress TS may be useful for the development of therapeutics for those whose cancers overexpress TS *de novo*. [© 2001 Lippincott Williams & Wilkins.]

Key words: COMPARE analysis, NCI Anticancer Drug Screen, thymidylate synthase, UNIDRUG.

Correspondence to AL Parr, National Cancer Institute, National Naval Medical Center, 8901 Wisconsin Avenue, Bldg 8, Rm 5101, Bethesda, MD 20889-5105, USA.
Tel: (+1) 301 496 0914; Fax: (+1) 301 496 0047;
E-mail: parra@mail.nih.gov

Introduction

Thymidylate synthase (TS) is a critical target for cancer therapeutics and represents the primary target for the fluoropyrimidine class of antineoplastic agents. TS catalyzes the methylation of deoxyuridylate to thymidylate which is required for the maintenance and replication of DNA. Elevated levels of TS have been demonstrated to be a common mechanism of insensitivity to both folate and nucleotide inhibitors of this enzyme. Recently, several clinical studies have found a relationship between the levels of TS and clinical outcome as measured by tumor response, thus supporting the critical role of TS as a target and as a determinant of drug sensitivity.[1,2] Of particular importance is the observation that high levels of intratumoral TS predict for a markedly diminished response rate to 5-fluorouracil (5-FU). In an investigation by Leichman and colleagues,[1] 41 patients with advanced gastrointestinal cancers were treated with a 5-FU-based regimen. The intratumoral TS levels in these patients were quantitated using an RT-PCR assay. Those patients with TS levels greater than 4 arbitrary units (AU) had only a 5% response rate to therapy compared with those less than or equal to 4 AU whom enjoyed a response rate of 56%. Furthermore, there was a significant survival advantage for those patients with low TS levels. In a subsequent study by Lenz and colleagues,[2] 36 patients with advanced colorectal cancer were treated with the regimen of 5-FU plus folinic acid. As in the Leichman study, the patients in this study whose intratumoral TS levels were less than 4 AU had a 60% response rate compared with 0% for those with levels in excess of 4 AU.

Given the above, we wanted to investigate the ability of the National Cancer Institute Anticancer Drug Screen (NCI-ACDS) database to identify compounds selectively cytotoxic to high TS-expressing cell lines. This involved measuring the TS expression levels in 53 human tumor cell lines used in the screen and then using the pattern of expression to find compounds with correlated cytotoxicity or growth inhibition effects. The intracellular level of TS protein in each of the 53 cell lines was measured using Western immunoblot (Table 1). The database of compounds previously screened in the NCI assay was searched for agents having relatively high cytotoxic or growth inhibition activity in the high TS-expressing cell lines. First, the 'molecular target' version of the COMPARE program[3,4] was used to identify candidates for validation. Then, when validation of the COMPARE-selected compounds proved unsatisfactory, we used a cluster-analyzed version of the database (UNIDRUG) to select candidates. The association between TS expression level and toxicity was confirmed for selected members of one of the UNIDRUG clusters.

The ultimate goal of this work is the identification of agents that would be active in patients where the available TS inhibitors are known to have limited clinical activity, i.e. in cancer cells with high TS levels.

Table 1. TS levels of cells from the NCI cell line screen was assayed from cell pellets provided by the Developmental Therapeutics Program and local cell cultures (TS levels were determined by Western blotting)

	Cell line	TS protein levels (AU)
NSCLC	NCI-H460	4.1
	HOP 92	1.0
	HOP 62	4.1
	EKVX	2.7
	NCI H226	8.8
	NCI-H23	4.3
	A549	4.7
	H157[a]	24.0
	H2122[a]	3.9
Colon	COLO-205	1.1
	HCC-2998	3.1
	SW620	11.7
	HCT 15	11.8
	HT-29	1.7
	KM12	2.8
	HCT-116	16.5
	H630-wt[a]	10.0
	H630-R10[a]	84.8
Renal	CAKI-1	8.4
	ACHN	0.9
	A498	0.5
	786-0	21.1
	UO31	2.9
	TK10	15.5
	RXF-393	3.0
Prostate	DU-145	3.5
	PC3	2.9
Leukemia	RPMI 8226	4.2
	HL-60	29.5
	MOLT-4	27.6
	K-562	4.9
CNS	SNB-19	1.9
	SF-295	8.5
	SF539	1.0
	U-251	7.1
	SF268	21.1
	SNB 75	0.2
Ovarian	SKOV-3	2.9
	OVAR 3	1.7
	I-GROV-1	9.2
	OVARC-4	3.3
	OVCAR-5	1.6
	OVCAR-8	5.4
Breast	BT549	4.4
	MDA-MB-45	13.2
	MDA-N	12.3
	HS578T	2.1
	T-47D	2.5
	MDA-MB-231/ATCC	4.2
	MDA-MB-468[a]	27.1
	1-B6[a]	17.6
	1-B6 with zinc[a]	78.0
Melanoma	SK-MEL-2	1.8
	UACC 257	7.9
	SKMEL-28	4.0
	MALME-3M	3.6
	UACC-62	7.4
	SK-MEL-5	2.8
	LOXIMVI	2.7
	M14	8.3
Epidermoid	A431[a]	1.4

[a]Indicates cell lines not contained in the original 53 cell line screen, but used in the validation experiments.

Materials and methods

Cell line maintenance

MDA-MB468 (human breast cancer cell line), H2122 and H157 (human lung cancer cell lines), and A431 (human epidermoid carcinoma) cells are available from the ATCC (http://www.atcc.org) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal bovine serum and maintained in a humidified

37°C incubator containing 5% CO_2. The H630 and H630-R10 cells were developed at the NCI[5] and maintained in DMEM with 10% dialyzed FBS. The 3-6 control and 1-B6 TS-overexpressing cells were developed in our laboratory by stable transfection of vector pM-TS into the MCF-7 human breast cancer cell line. The vector carries a zinc inducible metallothienine promoter. These cell lines were maintained in RPMI medium supplemented with 10% FBS and 30 μg/ml G418.

Toxicity studies

Experimental compounds were obtained from the repository of the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI (Table 2). All compounds were dissolved in DMSO. Cells were seeded in 96-well plates at 1500–4500 cells/well and allowed to grow for 48 h, at which point the media was removed and replaced with media containing varying concentrations (0–100 μM) of the experimental compounds and incubated for 72 h at 37°C. In the case of the 3-6 and 1-B6 cells, 42 h after being plated, the media was removed and replaced with media containing 100 μM $ZnCl_2$. After a 6-h incubation at 37°C, the $ZnCl_2$-containing media was removed, the cells were washed twice with regular media and the cells treated with the compounds as described above.

Table 2. Compounds tested in the MTS assay: these compounds were tested based on their selection by a modified COMPARE analysis (CEC) correlating their toxicity to high TS-expressing cells

CEC (PCC for cluster)	NSC no.	PCC
GRVA260 (0.423)	382053	0.50
	127755	0.44
GRVA242 (0.371)	170896	0.54
	17275	0.43
GRVA637 (0.365)	3578	0.50
GRVA240 (0.354)	681279	0.51
GRVA461 (0.344)	653840	0.58
	659610	0.52
GRVA78 (0.329)	667058	0.57
GRVA262 (0.308)	255917	0.57
GRVA261 (0.298)	8806	0.48
GRVA226 (0.272)	640642	0.54
GRVA16 (0.271)	615830	0.51
GRVA583 (0.271)	689959	0.51
GRVA74 (0.271)	666074	0.47
GRVA589 (0.270)	624192	0.54
GRVA17 (0.267)	53183	0.51
	667046	0.47
GRVA18 (0.261)	670995	0.46
GRVA105 (0.250)	640526	0.48
GRVA628	152731	0.63
	3088	0.39

MTS assay

Viability was determined using the Cell Titer 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). Following the 72 h exposure to the compounds, the kit's MTS solution was mixed with PMS solution in a 20:1 ratio and added directly to each well of the plate. The plates were incubated at 37°C for 30 min to 4 h. When an adequate color gradient had developed, the plates were read at 490 nm with a microtiter ELISA plate reader.

Western blot analysis

Frozen cell pellets representing each line in the 53-cell line screen were provided by the Developmental Therapeutics Program. Pellets for MDA-MB468, H122, H157, A431, H630, H630-R10, MCF-736 and MCF-71B6 were harvested from our laboratory stocks. Pellets were lysed by resuspension in 0.05 M KH_2PO_4 buffer, pH 7.2, followed by sonication and centrifugation at 15 000 *g* for 15 min at 4°C. Protein concentrations were determined using the BioRad protein assay kit.[6] Total protein (100 μg) from each cell line was resolved on a 12.5% polyacrylamide gel according to the method of Laemmli.[7] The gels were transferred onto nitrocellulose membranes (Schleicher & Schull, Keene, NH) and incubated in blotto (5% instant nonfat milk, 10 mM Tris, 0.01% thimerosal) for 30 min to block non-specific binding sites. The membranes were incubated with TS106 primary antibody against human TS[8] at a 1:200 dilution for 90 min, washed with PBS/0.1% Tween 20, followed by a 30-min incubation in goat anti-mouse horseradish peroxidase-conjugated secondary antibody (BioRad, Hercules, CA) at a 1:100 dilution. After washing with PBS/0.1% Tween, the protein bands were visualized using the ECL method (Amersham, Little Chalfont, UK). The resulting blots were scanned using a Microtech Scanmaker III and protein amounts were determined by scanning densitometry using NIH Image software (version 1.6) developed by Wayne Rasband, NIMH. To normalize protein levels across all blots relative to each other, α-tubulin protein was detected on each blot and levels adjusted accordingly to account for loading differences. Each blot also contained a lane with an equal amount of H630-wt cell lysate. The TS signal from this cell line was assigned an arbitrary value of 10. All other bands were assigned a relative value based on comparison to this control, thereby normalizing the protein amounts across all blots.

Unidrug pattern data set

In order to reduce the bias in the drug screen database toward the overrepresentation of some chemical structure and activity classes, a *k*-means algorithm-based clustering process was developed (T Myers, unpublished). In brief, a series of iterative clustering optimizations was performed on the NCI-ACDS IC_{50} patterns to find 1200 clusters that were well populated but maximally diverse. Exemplar compounds, non-confidential compounds closest to the center of each cluster, were taken to represent the entire database. In theory, and as first demonstrated by early applications of the COMPARE program and many examples since, highly correlated groups of compounds will share the same physicochemical properties, and, more importantly, often share mechanism of action properties. This phenomenon was named by the late Ken Paull the 'COMPARE effect'. Appropriately, the sets of compounds represented by the database exemplars are termed COMPARE Effect Clusters (CECs). When a 'molecular target' pattern comprising some cell characteristic measured in the NCI-ACDS is used to search for correlating activity patterns, a search against the redundancy-reduced set of CEC exemplars is expected to provide a more concise and accurate survey of the molecular target's role in measured cytotoxicity. Validation of members of the cluster, optimally without regard to the correlation of the individual compound with the molecular target, provides strong evidence for the involvement of the factor of interest (or another factor that is well correlated with the factor of interest) in the pharmacological mechanism of the group.

Results and discussion

Based on the molecular target COMPARE analysis, 12 compounds with the highest Pearson correlation coefficients (ranging from 0.655 to 0.781) were tested using the MTS assay in cell lines that had relatively high (H157 and MDA) or low (A431 and H2122) TS levels. These four cell lines were chosen for validation because they were not included in the 53 cell line screen used to generate the correlation coefficients and they have large differences (5- to 10-fold) in their expression levels of TS (Table 1). None of the tested compounds identified by the molecular target COMPARE analysis demonstrated selectivity against the two high TS cell lines.

Since the molecular target COMPARE analysis failed to yield selective compounds, a different analytical approach was followed. The modified analysis differs from the traditional COMPARE in that the correlation of measured factor, in this case TS expression, with the screened compounds is calculated for individual compounds (as does COMPARE) but evaluated for groups of compounds instead of simply ranking individual compound correlation results. These groups, termed CECs, are created independently to represent groups of compounds having a similar mechanism of cytotoxic or growth inhibitory action. The relatedness of a CEC to a molecular target is then considered on the basis of the correlation of a centroid pattern or single exemplar pattern, as well as the range of correlations for individual members of the cluster.

One representative compound with the highest correlation coefficient from each of 16 CECs identified by this second analysis was tested in the two high and two low TS-expressing cell lines. NSC 382053, which belonged to the mechanism of action group with the highest correlation with TS levels, appeared to be selectively toxic against the two high TS-expressing cell lines as compared to the two low TS lines (Figure 1a). A second compound from this CEC, NSC 127755 (triazine antifol), was tested against these same four cell lines and appeared to be selectively toxic against the two cell lines having the highest TS levels (Figure 1b). None of the compounds tested from any of the other CECs appeared to have selective toxicity against the four cell lines used for validation.

Two other compounds, trimetrexate (NSC 352122) and melphalan (NSC 8806), were also tested in the four cell line validation set. Trimetrexate was tested because its mode of action was similar to that of the compounds from the CEC containing NSC 382053 and NSC 127755 (DHFR inhibitors) which had the highest correlation coefficients and contained the only compounds that maintained selectivity for TS overexpression in the subsequent four cell line validation test. In addition, trimetrexate is an FDA approved agent for the treatment of refractory *Pneumocystis carinii* pneumonia. Melphalan was chosen for further testing since it had the highest Pearson correlation coefficient relative to TS levels in the cell line screen of any of the standard agents available for clinical use. The cytotoxicity studies for trimetrexate (Figure 1c) and melphalan (Figure 1d) did not demonstrate selectivity for the TS-overexpressing cell lines.

To further explore the relationship between TS overexpression and the potential selective cytotoxicity against cell lines with TS overexpression, we tested the cytotoxicity of each of the four compounds in two additional model systems. In these additional models, TS overexpression was 'forced' by either transfection or by prior exposure to TS inhibitors. We used these

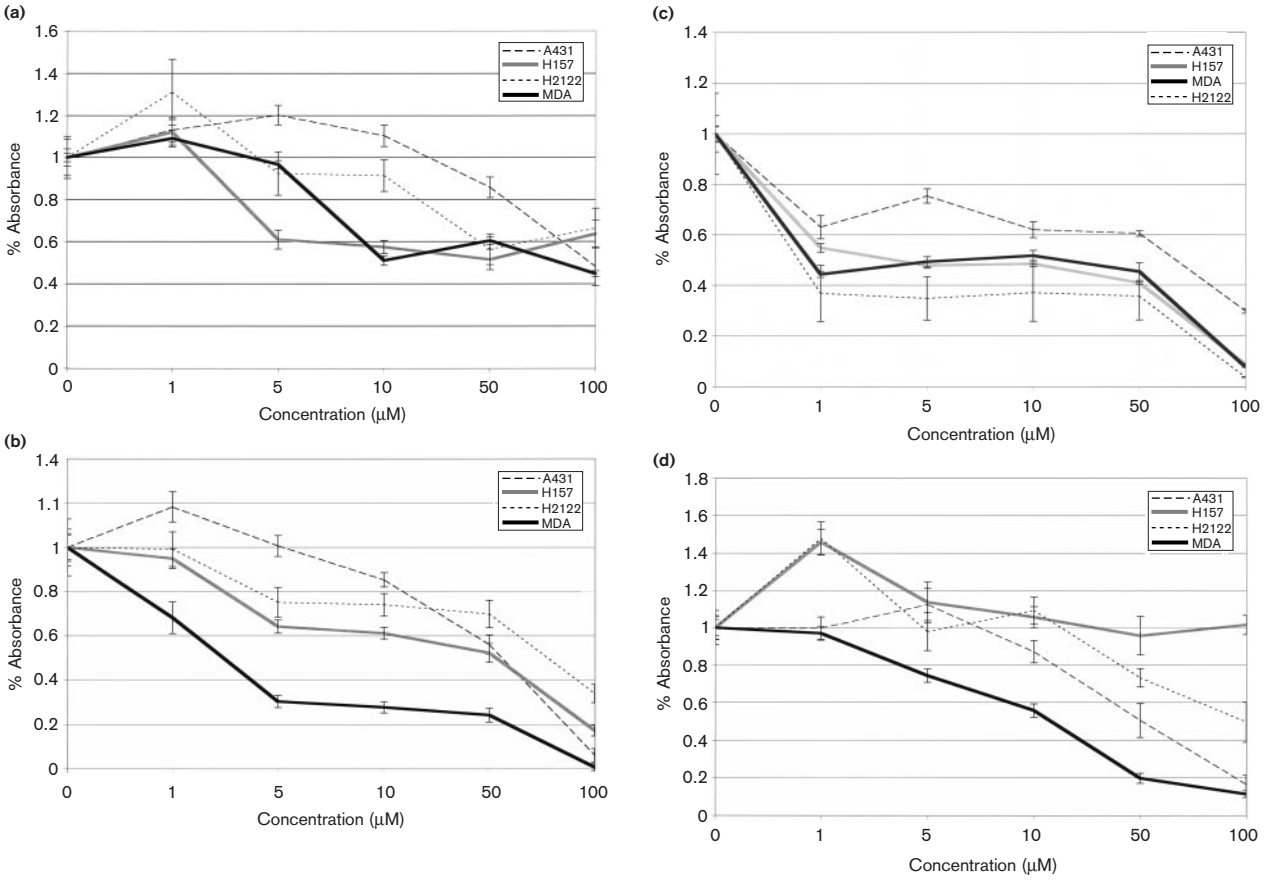

Figure 1. (a–d). Cell death curves generated by the MTS assay. For (a–d), cell lines expressing low levels of TS (A431 and H2122) along with cell lines expressing high levels of TS (MDA-MB 468 and H157) were tested with compounds chosen by the CEC analysis (a, 382053; b, 127755; c, 352122; d, 8806) for 72 h and then assayed by the MTS assay.

additional models to test whether the apparent selectivity was the direct result of TS overexpression or simply an association with TS overexpression and thus an indirect consequence of TS overexpression. Four compounds (NSC 382053, NSC 127755, trimetrexate and melphalan) were tested against the human colon carcinoma cell line H630-wt versus H630-R10 and 3-6 versus 1-B6 MCF-7 human breast cancer subclones. In the case of H630-R10 cells, the TS was overexpressed due to step-wise growth in increasing concentrations of 5-FU. These cells are known to have 8.4-fold more TS when compared with the parental cell line due to stable gene amplification. The stable TS transfected MCF-7 breast cancer cells line overexpresses TS by about 4.4-fold compared with the empty vector containing control line when exposed to zinc. None of the four compounds demonstrated significant differences in toxicity between high and low TS-expressing cells in either model system (data not shown).

In this study, we selected compounds from the NCI repository that exhibited the greatest degree of relative selective cytotoxicity against those cell lines contained in the NCI's cell line drug screen that expressed the highest levels of TS protein. The first group of compounds tested was selected by using a traditional COMPARE algorithm that identifies individual compounds with the highest correlation coefficient with respect to cellular TS protein levels. Since this algorithm failed to identify TS-selective compounds that passed the four cell line validation test, we exploited a clustered version of the NCI screen database, UNIDRUG, in an attempt to select compounds based on their grouping by mechanism of action, thus increasing confidence by simultaneously assessing multiple compounds belonging to a given group rather than using correlations based on individual compounds. Based on this latter algorithm, the mechanism of action groups with the best correlation coefficients included compounds that represented DHFR inhibitors and alkylating agents.

The initial screen of compounds was carried out in the cell lines that have 'naturally' high or low TS levels.

The best compounds and their mechanism of action groups identified by this initial screen were next tested in four additional cell lines not contained in the initial cell line screen as a validation test. Two of these four cell lines had relatively high and two low TS protein expression (Table 1). Of the 16 CECs tested, only the group with the highest correlation demonstrated selectivity against TS-overexpressing cell lines in the four cell line validation test. Trimetrexate and melphalan, representing additional members of the best clusters, were tested because of their clinical availability and/or membership in the CECs with the highest correlations with TS levels. These drugs failed to demonstrate selectivity in the validation test.

The two compounds consistently found to be selectively effective against the 'naturally' high TS cell lines as well as trimetrexate and melphalan were then tested in cell lines induced to overexpress TS by either stable and inducible transduction (human MCF-7 breast cancer cell line) or from stable TS gene amplification resulting from stepwise increasing exposures to 5-FU (H630-R10 human colon cancer cell line). In these induced TS models, none of the compounds demonstrated selective cytotoxicity. These results suggest that the compounds were most likely not selectively killing cells through a direct or indirect TS-mediated mechanism. Rather, the relationship between TS expression and cytotoxicity exhibited by the compounds having the best correlation was one of an association whereby TS was acting as a 'marker' for selectivity. The lack of a direct relationship between TS and the DHFR inhibitors is somewhat surprising given the known role of TS in antifolate sensitivity,[9] wherein low TS levels have been associated with insensitivity to DHFR inhibitors. Nonetheless, these data support a role for the identified compounds as potentially useful drugs for the treatment of cancers that overexpress TS protein *de novo* as is the case for more than half of patients with advanced colorectal.[1,2] It is unlikely that these compounds would be useful for the treatment of patients who become refractory to TS inhibitor-based therapy by virtue of TS amplification, which is probably one of the more common mechanisms of clinical 5-FU insensitivity.

In summary, we have identified compounds that demonstrate modest cytotoxic selectivity in cell lines having relatively high TS protein levels. This selectivity is indirectly related to TS protein level, which appears to be a marker for selectivity rather than the cellular target for the identified compounds. It is possible that the identified compounds may have value in the therapy of patients whose cancers demonstrate *de novo* high levels of TS.

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