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Pharmacological and Toxicological Evaluation of a New Series of Thymidylate Synthase Inhibitors as Anticancer Agents

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Abstract. *Thymidylate synthase (TS) is responsible for catalysing the de novo biosynthesis of doexythymidine monophosphate and is a target for many anticancer drugs. A series of thymidylate synthase inhibitors (TSIs), synthesised in our laboratory, were submitted to primary anticancer screening by the National Cancer Institute (NCI). Four compounds, 3,3-bis(4-methoxyphenyl)-1H,3H-naphtho[1,8-cd]pyran-1-one (MR7), 6-chloro-3,3-bis(4-hydroxyphenyl)-1H,3H-naphtho[1,8-cd]pyran-1-one (MR21), 3,3-bis(3-fluoro-4-hydroxyphenyl)-1H,3H-naphtho[1,8-cd]pyran-1-one (MR35) and 6-bromo-3,3-bis(3-chloro-4-hydroxyphenyl)-1H,3H-naphtho[1,8-cd]pyran-1-one (MR36), passed the criteria and were automatically scheduled for evaluation against the full panel of 60 human tumour cell lines. In this study, the antiproliferative activity of the substances against SK-MEL-2 cells (from metastatic tissue) and SK-MEL-28 cells (from primary malignant melanoma cells) was investigated. Neutral Red uptake and the MTT test were performed to confirm the results of the NCI, and [^{3}H]-thymidine incorporation was performed as a test of the proliferation rate. Our results indicated that compounds MR21 and MR36 were the most active agents and the [^{3}H]-thymidine test was the best in predicting toxicity against melanoma cells.*

Thymidylate synthase (TS), a crucial enzyme for DNA synthesis, is responsible for catalysing the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by a reductive methylation involving *N*5-*N*10-methylentetrahydrofolate (mTHF) as a cofactor. Because of its central role in maintaining adequate levels of dTMP for DNA synthesis and repair, TS is an important target for cancer chemotherapy (1, 2). The best-known classic inhibitors are structurally related to substrates, such as 5-Fluorouracil or to cofactors such as tomudex or methotrexate, but the similarity in their chemical structures often induces resistance; therefore, new inhibitors must be proposed as drug candidates. With the aim of designing new molecules that could maintain the antifolate activity with respect to the TS enzyme and overcome resistance, molecules with structures markedly unrelated to the classic inhibitors were obtained through a structure-based drug design approach (3). Among them, the best inhibitors were naphthalic anhydride derivatives. The compounds were submitted to a primary *in vitro* anticancer screening by the National Cancer Institute (NCI, Bethesda, USA). Four compounds, MR7, MR21, MR35 and MR36 (Figure 1), passed the criteria for anticancer activity and were automatically scheduled for evaluation against the full panel of 60 human tumour cell lines. NCI has implemented a large scale *in vitro* drug-screening program that requires a very efficient automated assay of drug effects on tumour cell viability or growth (4, 5). The cytotoxicity of the compounds was evaluated using the sulforhodamine B protein assay (SRB) (6).

The four naphthalein derivatives, MR7, MR21, MR35 and MR36, induced a dose-dependent growth inhibition of NCI-H460 (lung cancer) cells, MCF-7 (breast cancer) cells, SF-268 (central nervous system cancer) cells and melanoma (UACC-62) cells, to different degrees. Cutaneous malignant melanoma accounts for 10% of skin cancer; this aggressive tumour is characterised by constitutive chemo-resistance to the usual chemotherapy protocols (7), but the above-mentioned melanoma cells responded to treatment with the four naphtahlein derivatives. The aim of this work was to investigate the cytotoxicity of the new thymidylate synthase

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Key Words: Thymidylate synthase, cytotoxicity, cell proliferation, SK-MEL-2, SK-MEL-28.

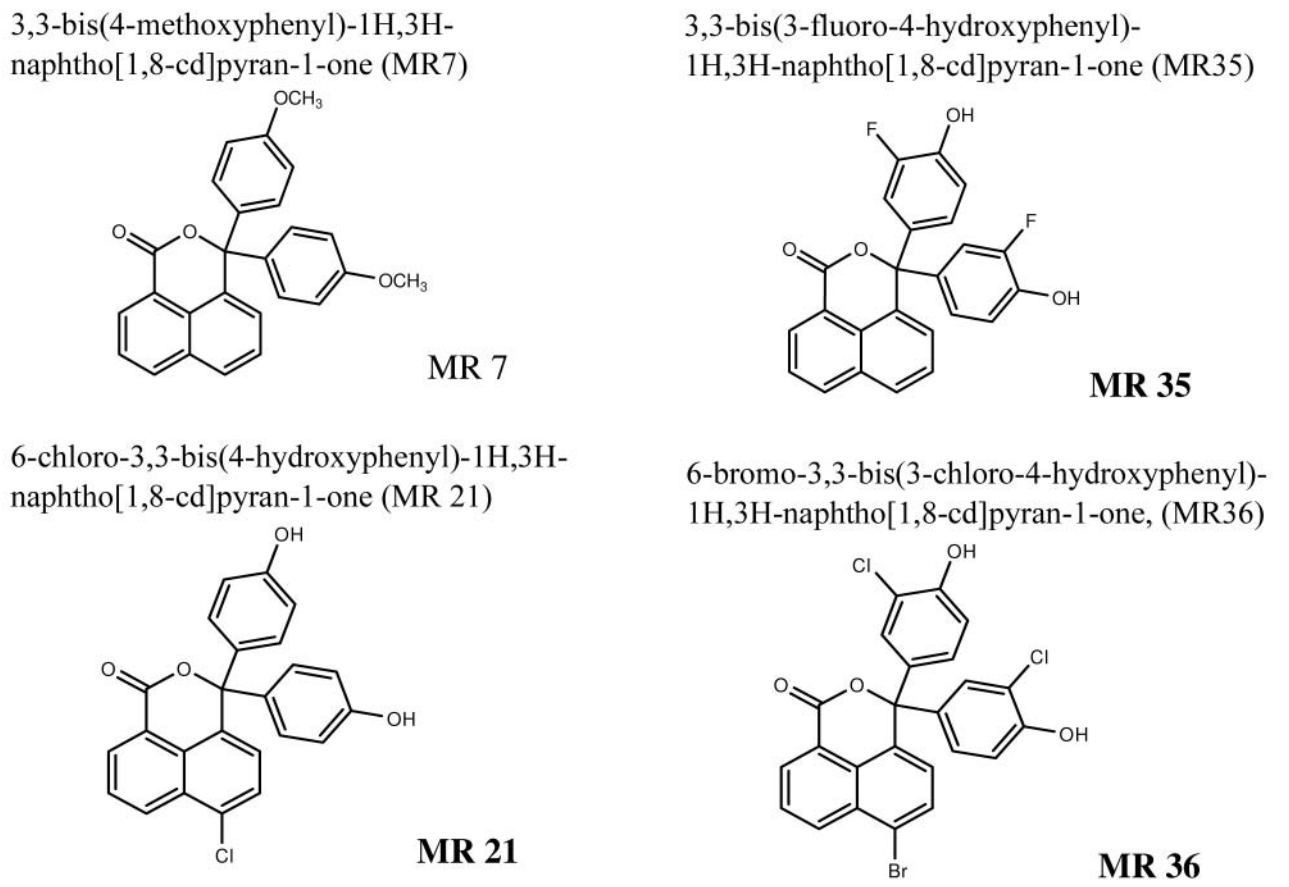

Figure 1. *Chemical structures of the new thymidylate synthase inhibitors (TSIs) synthesised.*

inhibitors on SK-MEL-2 and SK-MEL-28 cells by the Neutral Red uptake (NRU) and the MTT tests and the [^{3}H]-thymidine uptake assay, in addition to the tests performed by the NCI in order to confirm their activity as anti-proliferative agents.

Materials and Methods

Compounds. The four thymidylate synthase inhibitors (TSIs), MR7 ($C_{26}H_{20}O_4$), MR21 ($C_{24}H_{15}ClO_4$), MR36 ($C_{24}H_{13}BrCl_2O_4$) and MR35 ($C_{24}H_{14}F_2O_4$), were tested.

Cell culture. SK-MEL-2 cells, derived from metastatic tissue and SK-MEL-28 cells, derived from primary malignant melanoma cells, were used and cultured in MEM enriched with 10% foetal bovine serum (FBS), 1% antibiotic solution (penicillin 50 U/ml and streptomycin 0.5 mg/ml) and 1% L-glutamine. F75 flasks containing the cells were maintained at 37˚ C and 5% CO_2. Once cell confluence had been reached, the SK-MEL-2 and SK-MEL-28 cells were transferred, under sterile conditions, into 96-multiwell plates (100,000 cells/well for each line) for the toxicity tests. On the basis of NCI's results, the naphthalein derivatives, previously dissolved in MEM and DMSO (DMSO final concentration <0.1%), were added to the cultures at four different concentrations ranging from 0.001 μM to 100 μM and were incubated again under the same conditions for 48 h.

Cytotoxicity tests. The NRU and MTT tests and the [^{3}H]-thymidine incorporation assays were performed in our laboratory, while the SRB was performed by the NCI (8-10).

MTT test. In the MTT assay, a yellow tetrazolium salt is reduced to a blue formazan product by mitochondrial succinic dehydrogenase present only in metabolically active cells. The reaction was carried out *in situ* in multiwell plates and the reaction product was measured colorimetrically at 540 nm using a Bio-Rad 550 microplate reader (11).

NRU test. The NRU test is a cell survival/viability test based on the ability of viable cells to incorporate and bind neutral red, a weak cationic supravital dye that readly penetrates the cell membrane by non-ionic diffusion (12). The cell viability was determined by comparing the absorbance values of the 48-h-treated cells with those obtained from the controls, which were taken as 100% cell viability (13, 14).

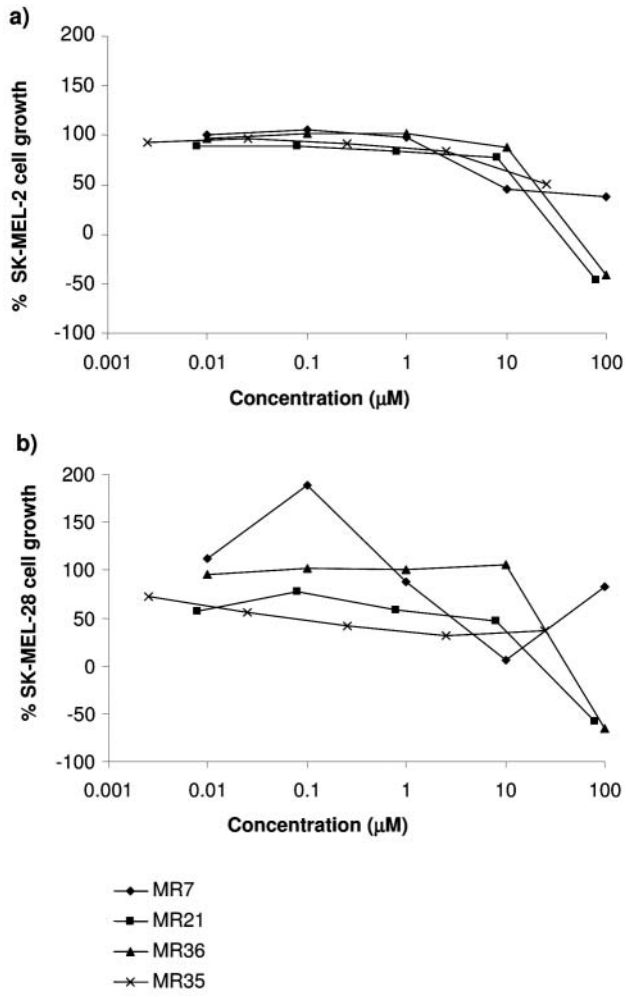

Figure 2. *Dose response curves were obtained from the results of the NCI screening test for SK-MEL-2 (a) and SK-MEL-28 (b) cell lines. Data are evaluated with respect to the total cell growth = 100%. +50% = growth inhibition 50; 0% = cytostatic effect and (TGI) and -50% =cytotoxic effect (LC50). The TSI concentrations are expressed in logarithmic scale.*

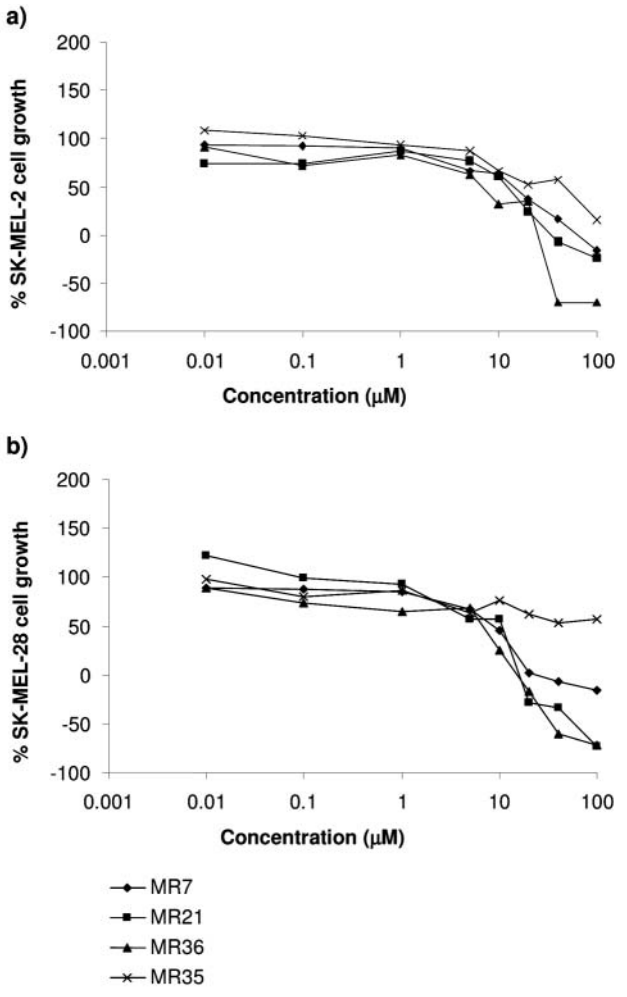

Figure 3. *Neutral Red uptake test results on SK-MEL-2 (a) and SK-MEL-28 (b) cells treated with the TSIs. The correlation between the percentage of growth and the TSI concentrations are expressed in logarithmic scale.*

[^{3}H]-Thymidine incorporation assay. This test allows the rate of cell proliferation to be measured after treatment with the compounds under study. The treatment effects were quantitated in the thymidine incorporation assay (TIA) by measuring the inhibition of DNA synthesis by the proliferating cells. Ninety-six-well tissue culture plates were used (inoculum: $7x10^5$ cells/well). After 48 h, each well was supplemented with 5 mCi [^{3}H]-thymidine. The assessment of radioactive nucleotide incorporation was measured in a scintillation β-counter (15). All the tests were carried out in triplicate.

Results

NCI data. Compounds were subjected to cytotoxicity tests by the NCI using five concentrations at ten-fold dilutions: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M (16). Cell viability was determined by the SRB assay. The NCI screening data report is collected against each cell line and analyses three different parameters: the growth inhibitory effect (GI_{50}=50% growth inhibition), the cytostatic effect (TGI=total growth inhibition) and the cytotoxic effect (LC_{50}=50% lethal concentration) (17). Analysis of the NCI results led to the following observations: the MR36 GI_{50} value indicated the high toxicity of the compound. Colon cancer and leukemia cells were the most sensitive to MR7 activity and MR21 displayed a good activity against colon cancer cell lines, while MR35 seemed to be the least active and least toxic compound (data not shown). The NCI results concerning the new TSI activity on SK-MEL-2 and SK-MEL-28 cells (Figure 2) indicated that the SK-MEL-28

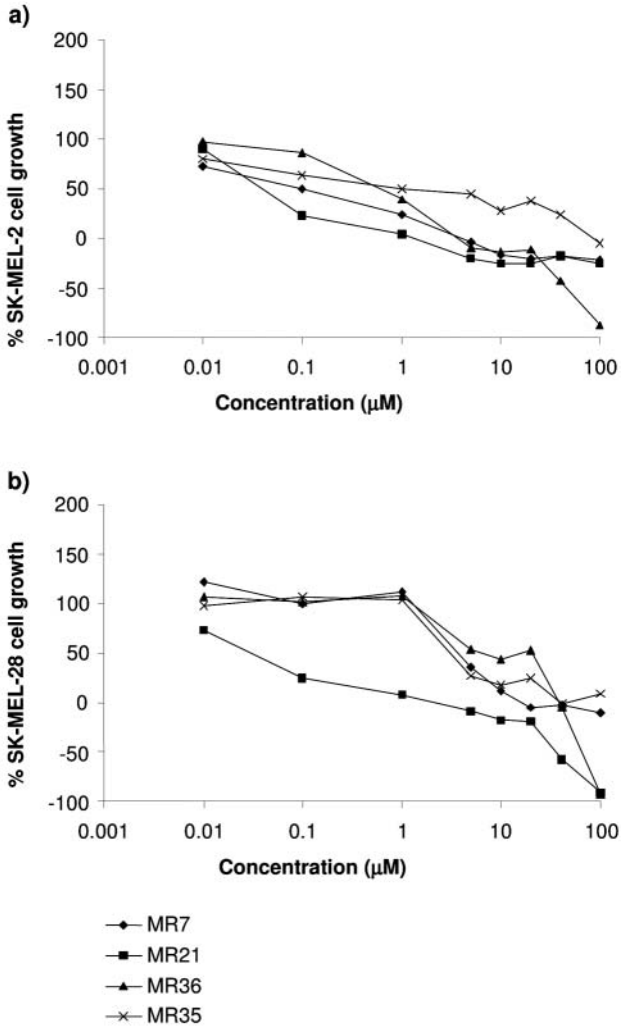

Figure 4. *MTT test results for the SK-MEL-2 (a) and SK-MEL-28 (b) cell lines treated with the TSIs for 48 h. The results take into consideration the growth inhibitory potency GI_{50}, the cytostatic effect (TGI) and the cytotoxic effect (LC_{50}). The concentrations are expressed in logarithmic scale.*

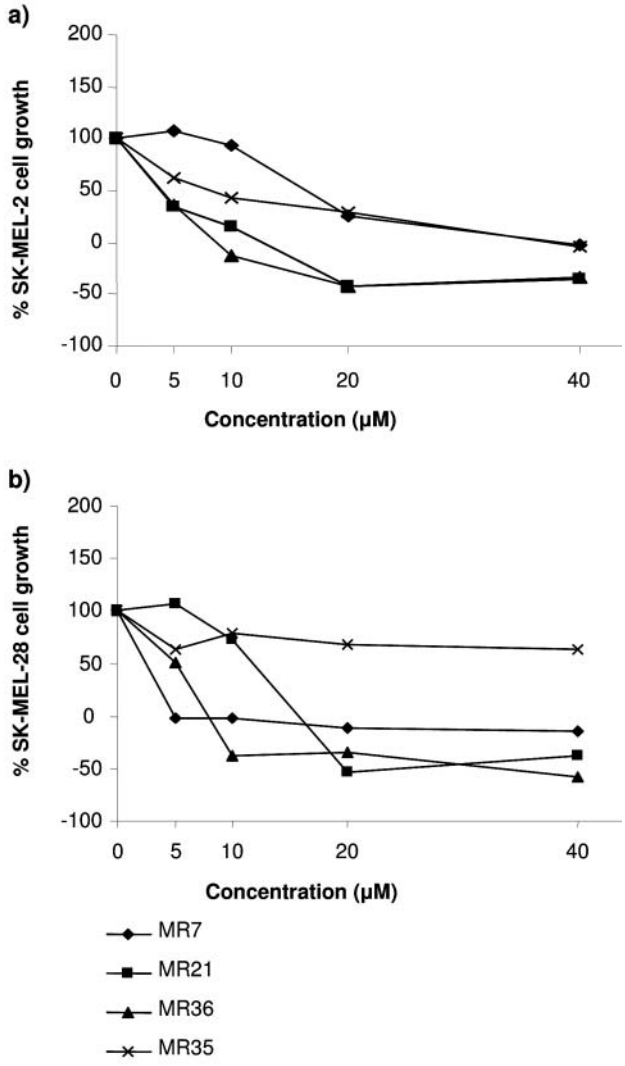

Figure 5. *[^{3}H]-Thymidine incorporation test results on SK-MEL-2 (a) and SK-MEL-28 (b) cell lines after treatment (48 h) with increasing concentrations of TSIs.*

cells were more sensitive to MR21 and MR35 than to SK-MEL-2, with a GI_{50} concentration of 0.01 μM for the two compounds on the SK-MEL-28 cells, and of 10 μM on the SK-MEL-2 cells, while MR36 did not modify cell proliferation. Finally, according to the NCI's results, MR21 appeared to be the most cytotoxic compound.

Cell viability assays. The NRU, MTT and [^{3}H]-thymidine incorporation assays were performed to study cell viability and DNA active synthesis. The dose-response cytotoxicity curves, as quantified by the NRU test (Figure 3), after exposure to the TSIs (48 h) clearly showed an almost identical dose-effect relationship for all the compounds on both the cell lines (with the exception of the highest concentration).

The results obtained from the MTT test, in contrast to the SRB data, were dose-dependent (Figure 4). MR21 was confirmed as the most cytotoxic compound for both the SK-MEL-2 and SK-MEL-28 cell lines (TGI=1μM and LG_{50}=40 μM only for SK-MEL-28); MR36 exerted a more toxic effect on SK-MEL-2 cells (LG_{50}=1 μM and TGI=4.2 μM). The MR7 activity was similar to that of MR21 and MR36 only on the SK-MEL-2 cells. SK-MEL-28 cell viability was the same regardless of treatment with either MR7 or MR35.

[^{3}H]-Thymidine incorporation assay. This assay was performed by treating the cells with the TSIs at four concentrations (from 5 μM to 40 μM), chosen on the basis

of their activity on cell proliferation. The dose-response cytotoxicity curves after exposure (48 h) of cells to MR7, MR21, MR36 and MR35 are shown in Figure 5. Determination of cell proliferation by [^{3}H]-thymidine uptake based on DNA synthesis emphasised the differences between the four compounds. MR36 was found to act mainly on the S-phase of the two cell lines to a greater extent than the other compounds. Surprisingly, MR7 cytotoxicity was evident on the SK-MEL-28 cell line at all the employed concentrations, while the TGI of the SK-MEL-2 cells was reached only with MR7 at 40 μM. Low concentrations of MR21 resulted in different toxicity on the two cell lines, while MR21 at 20 μM and 40 μM was toxic for both cell lines. MR35 displayed a cytotoxic effect mainly on the SK-MEL-2 cells.

Discussion

A novel class of TSIs were designed and synthesised as antimetabolites of TS. These compounds, MR7, MR21, MR35 and MR36, interfere with DNA synthesis competing with TS activity. In our *in vitro* experiments, the new TSIs competed with the folate to form an enzyme-inhibitor complex and to prevent the binding of the cofactor (mTHF) (3). Their action prevented the biosynthesis of pyrimidine nucleotides by interferring with cellular activities leading, finally, to cell death.

The first part of the present study reported the results obtained by the NCI in a large scale screening (60 cell lines). In the second part of the study, the *in vitro* toxicity of these compounds on SK-MEL-2 and SK-MEL-28 cells were evaluated by the NRU, MTT and [^{3}H]-thymidine incorporation assays. Finally, our data were compared with those obtained from the SRB assay performed by the NCI on the same cell lines (18).

The NCI results regarding the melanoma cell lines indicated that the compounds were able to interfere with cell growth and, in particular, the effect was more evident when MR21 and MR36 were added to SK-MEL-28 cultures. NCI anticancer drug screening resulted in similar toxicity patterns for SKMEL-2 when compared to the NRU performed in our laboratory, while the toxicity profile against SK-MEL-28 was slightly different. Using the NRU test, SK-MEL-28 appeared to be less sensitive and only showed signs of damage at high concentrations. NRU is a useful tool that detects the uptake of the dye by functional lysosomes, but this kind of damage is probably a late sign of cellular damage.

The MTT test is known to be more sensitive in detecting early toxicity (19) and is an index of cellular metabolic activity, mainly based on the enzymatic conversion of MTT in the mitochondria. Results from the test showed that MR21 at 40 μM reduced the cell growth (50%) of both cell lines. MR36 was confirmed to be the most toxic compound.

Finally, the [^{3}H]-thymidine incorporation assay evaluated the replicative cell capability. TS inhibition induced a specific block of DNA synthesis and, therefore, of the replicative cell activity. S-phase arrest normally precedes cell death and consequently the [^{3}H]-thymidine test provides information regarding cell damage at the earliest point in the cell cycle.

The MTT test and the [^{3}H]-thymidine incorporation assay showed that the primary melanoma SK-MEL-28 cell line was more susceptible to TSI action than was SK-MEL-2. The latter cells were derived from metastatic tissue and are subject to alterations in gene expression; these changes select clones that are more resistant to pharmacological treatment and could justify the different sensitivity towards the new TSIs. Moreover, the chemical structure of the compounds aided in interpreting our results: MR7 has two phenolic functions (Ar-OCH_3) which are blocked and interact with the active site of TS through a methyl group bound to the oxygen molecule. MR35 shows a significantly altered chemical reactivity when the hydrogen at position 5 of the pyrimidine ring is substituted by fluorine. Fluorine has an inductive effect, which is reflected in a much lower pKa with fluorouracil-containing compounds with respect to the natural compounds. In addition, the carbon-fluorine bond is stronger than the carbon-hydrogen bond and is less susceptible to enzymatic cleavage. Thus, substitution of a halogen atom can produce a molecule that sufficiently resembles a natural cofactor to interact with the enzyme and form a highly stable compound, thereby increasing the molecular specificity.

This study was designed to compare a preliminary test assessed by the NCI screening assay and three cell proliferation assays in order to test melanoma cell line sensitivity to antifolate agents. To our knowledge, this is the first reported direct comparison of sensitivity assays for antifolates on melanoma cell lines. There are many other assay systems available for drug sensitivity determinations, and we believe that these compounds deserve further study and testing of new analogues for a better understanding of their mechanism of action.

Over the last ten years, the design of potentially active drugs, i.e., antimetabolites, has been concentrated on structural changes of existing metabolites, such as DNA and RNA synthesis precursors. Our results outline the consequences of TS inhibition associated with cellular proliferation, with particular reference to apoptosis pathways and cellular cycle regulation.

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