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Thymidylate synthase as a target enzyme for the melanoma-specific toxicity of 4-S-cysteaminylphenol and *N*-acetyl-4-S-cysteaminylphenol*

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Summary. The rationale for melanoma-specific antitumor agents containing phenolic amines is based in part on the ability of the enzyme tyrosinase to oxidize these prodrugs to toxic intermediates. The phenolic amine compounds 4-S-cysteaminylphenol (4-S-CAP) and *N*-acetyl-4-S-cysteaminylphenol (N-Ac-4-S-CAP) inhibited in situ thymidylate synthase activity in pigmented melanoma cell lines but had little or no effect on nonpigmented and nonmelanoma cell lines. Theophylline, a cyclic adenosine monophosphate (cAMP) phosphodiesterase inhibitor, increased tyrosinase activity and potentiated the inhibition of in situ thymidylate synthase by N-Ac-4-S-CAP. The inhibition of in situ thymidylate synthase by both drugs in pigmented melanoma cells correlated with the inhibition of DNA synthesis and cell growth and was not due to an indirect effect caused by inhibition of the enzyme dihydrofolate reductase. 4-S-CAP inhibition of thymidylate synthase activity in cell free extracts required oxidation of the drug. In the presence of tyrosinase, the concentration causing a 50% inhibition of thymidylate synthase activity (IC_{50}) in cell-free extacts was <10 μM, but no inhibition was observed in its absence, even at a drug concentration of 500 μM. Two reducing agents, dithioerythritol and glutathione, effectively blocked the inhibition of thymidylate synthase by oxidized 4-S-CAP. In pigmented melanoma cells containing the enzyme tyrosinase, the quinone-mediated mechanism of inhibition of DNA synthesis via inhibition of thymidylate synthase may be uniquely important in the expression of phenolic amine cytotoxicity.

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Introduction

Melanospecificity and antimelanoma effects have been observed for a variety of phenolic amine compounds [1, 4, 14, 20]. 4-S-Cysteaminylphenol is cytotoxic in a heavily pigmented clone of the B16 melanoma cell line but not in the nonpigmented clone [30]. Various phenolic amine compounds are cytotoxic to follicular melanocytes of black C57BL/6J mice but not to melanocytes or keratinocytes of control albino follicles [13]. It has been postulated that 4-S-CAP is toxic to melanoma cells only after its oxidation by tyrosinase [30]. 4-S-CAP is a better substrate for tyrosinase than L-tyrosine [14] and is oxidized to the corresponding orthoquinone, which forms covalent bonds with proteins through cysteine residues [15]. Thus, the cytotoxicity of 4-S-CAP may result from conversion to the orthoquinone and subsequent conjugation with sulfhydryl-containing enzymes. The cytotoxic activity of 4-S-CAP is associated with inhibition of thymidine incorporation with the strongest inhibition being observed in heavily melanized melanoma cells [30]. On the other hand, uridine and leucine incorporation remain largely unaffected [30]. Taken together, these observations indicate that 4-S-CAP primarily exerts its cytotoxicity through inhibition of thymidine incorporation into DNA.

Thymidylate synthase [5,10-methylenetetrahydrofolate: deoxyuridylate (dUMP) C-methyltransferase, EC 2.1.1.45] is an enzyme that catalyzes the reductive methylation of dUMP to deoxythymidylate (dTMP), an essential precursor for DNA synthesis. Inhibition of thymidylate synthase significantly contributes to the cytotoxicity of many antitumor agents, e.g., 5-fluoropyrimidines and antifolates [8, 16]. Inhibition of dTMP synthesis leads to a complex situation in which DNA synthesis is impaired but protein synthesis and RNA synthesis are not, a phenomenon termed "thymineless death" [5]. In the present study, an intact cell assay, that measures thymidylate synthase activity [29] was used to investigate the mechanism of cytotoxicity of 4-S-CAP and N-Ac-4-S-CAP. This assay enables the direct and rapid assessment of enzyme activity and inhibition by cytotoxic agents. Its advantage over cell-

free systems is that the cell membrane, multienzyme complexes, and network of metabolic pathways and their control within the cell remain unperturbed [29]. 4-S-CAP and N-Ac-4-S-CAP were tested for their ability to inhibit in situ thymidylate synthase activity in melanoma cell lines showing varying degrees of tyrosinase activity and in non-melanoma cell lines.

Materials and methods

Chemicals. Dithioerythritol (DTE), glutathione (GSH), L-cysteine, tyrosinase, 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), tetrahydrofolic acid, and [5-^{3}H]-deoxyuridine ([5-^{3}H]-dUrd, 20.4 Ci/mmol) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Theophylline, 4-hydroxythiophenol, and 2-methyl-2-oxazoline were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). [5-^{3}H-Deoxycytidine ([5-^{3}H]-dCyd, 24.2 Ci/mmol), [3,5-^{3}H]-tyrosine (50 Ci/mmol), and [methyl-^{3}H]-thymidine ([5-^{3}H]-Thd, 6.7 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). [5-^{3}H]-Deoxyuridine monophosphate ([5-^{3}H]-dUMP, 20 Ci/mmol) was obtained from Amersham (Arlington Heights, Ill).

Synthesis of N-Ac-4-S-CAP and 4-S-CAP. N-Ac-4-S-CAP and 4-S-CAP were synthesized according to the methods of Padgette et al. [22]. For synthesis of N-Ac-4-S-CAP, a mixture of 2-methyl-2-oxazoline (2.11 ml, 24.7 mmol) and 4-mercaptophenol (3.11 g, 24.7 mmol) was heated under reflux for 2 h at 130°C in the presence of argon. After the reaction mixture had cooled, the oily residue was dissolved in 10 ml absolute ethanol and N-Ac-4-S-CAP was crystallized from dilute ethanol at 4°C as white crystals. Contaminants were removed by two extraction steps with ether followed by two with methylene chloride. For synthesis of 4-S-CAP, *N*-acetyl-cysteaminylphenol (2 g, 5.98 mmol) was refluxed in concentrated HCl (2.37 ml) for 12 h. After it had cooled to room temperature, the reaction mixture was diluted with H_2O (7 ml) and extracted twice with ether (7 ml), after which the aqueous phase was evaporated to dryness and then dissolved in H_2O (7 ml) and reevaporated twice. 4-S-Cysteaminylphenol was recrystallized in ethanol/ether as white crystals (1.4 g, 70%). The melting points for N-Ac-4-S-CAP and 4-S-CAP were 120° C and 125° – 127° C, respectively [22]. For confirmation of structures of N-Ac-4-S-CAP and 4-S-CAP, NMR spectra were obtained on a Bruker 300 MHz spectrometer (Model Ac300) using acetone-d_6 and D_2O, respectively, as solvents [22].

Cell lines. Human SK-MEL-28 melanoma, human ZR-75 breast carcinoma, human K562 myelogenous leukemia, human T-47D breast ductal carcinoma and human HTB-35 squamous carcinoma cell lines were purchased from the American Type Culture Collection (Rockville, Md.). The B16-BL6 and B16-F1 cell lines [7] were obtained from Dr. I. J. Fidler, M. D. Anderson Cancer Center (Houston, Tex.). The SK-MEL-30 cell line was a gift from Dr. M. Wick, Dana Farber Cancer Institute (Boston, Mass.). Cell lines were maintained as monolayers in 75-cm^2 plastic Falcon flasks containing RPMI-1640 medium supplemented with 10% fetal calf serum or 10% calf serum, 2 mM glutamine, 0.2 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 50 μg insulin/ml, 100 μg streptomycin/ml, 100 IU penicillin/ml, and 2.5 μg gentamicin/ml. All cultures were maintained at 37° C in a humidified incubator containing 5% CO_2 and 95% air.

Tyrosinase assay. The method used in the present study was a modification of the procedure described by Halaban and Lerner [11]. Culture medium was removed from 24-well microtiter plates containing cells in log-phase growth and replaced with 0.5 ml Hanks' balanced salt solution (HBSS) containing 0.5 μCi L-[3,5-^{3}H]-tyrosine that had previously been reduced to dryness under a stream of nitrogen gas. Following incubation for 60 min at 37° C with intact cells, the accumulation of tritiated water in 0.5 ml medium was measured. The buffered salt solution was removed from the wells and added to 1.5-ml microfuge tubes. To each tube was added 0.5 ml charcoal solution (300 mg charcoal in 10% trichloroacetic acid TCA), and the mixture was periodically shaken for 30 min prior to centrifugation at 10,000 rpm for 6 min. Aliquots of 0.5 ml were pipetted off and added to 6 ml Ultima Gold scintillation fluid (Packard Instrument Co., Downers Grove, Ill.). Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter (model 1900CA). All determinations were done in triplicate.

Preparation of cell-free extracts. Human K562 leukemia cells (~2×10^8) were harvested by centrifugation from 75-cm^2 culture flasks and washed twice with HBSS. Cells were resuspended in 1.5 vol. 20 mM TRIS buffer containing 1 mM DTE (pH 7.4). The resuspended cells were sonicated using a Sonics and Materials sonifier (model Vibra cell; Danbury, Conn.) run at a setting of 70 with 5 pulses of 20-s duration. The suspension was centrifuged at 15,000 rpm for 30 min and the pellet (containing) <1% of total enzyme activity) was discarded. The supernatant was decanted, aliquotted, and stored at –20° C until assayed for enzyme activity.

Assay of thymidylate synthase activity in cell extracts. Thymidylate synthase activity was assayed essentially as described by Roberts [25]. This sensitive procedure measures the release of tritium (as tritiated water) from the 5-position of [5-^{3}H]-dUMP during the formation of dTMP [18]. Briefly, 20 μl cell extract was mixed with 180 μl reaction mixture containing final concentrations of 0.15 M Trizma (pH 7.4), 60 mM NaF, 0.052% formaldehyde, 10 mM tetrahydrofolate, and 0.667 μCi [5-^{3}H]-dUMP. Radiolabeled dUMP had previously been reduced to dryness under a stream of nitrogen gas. The mixture was incubated at 37° C for 45 min. The reaction was terminated by the addition of 0.5 ml activated-charcoal suspension (300 mg charcoal in 10% TCA), which removed unreacted [5-^{3}H]-dUMP. The reaction mixtures were periodically vortexed for 30 min and then centrifuged for 10 min at 15,000 rpm. Aliquots of 0.5 ml were pipetted off and added to 6 ml Ultima Gold scintillation fluid, and the radioactivity was measured. All reactions were done in duplicate.

In situ thymidylate synthase assay. In situ thymidylate synthase activity was assayed by a modification of the procedure described by Yalowich and Kalman [29], which measures tritiated water generated from [5-^{3}H]-dCyd. In this assay, cell lines were seeded at 7.5×10^4 cells/well and incubated for 24 h in Falcon 24-well flat-bottom microtiter plates containing complete RPMI-1640 media supplemented with 10% fetal calf or calf serum. Cells were then incubated for 2 h at 37° C with selected concentrations of 4-S-CAP or N-Ac-4-S-CAP (2 mM – 100 μM) in complete media supplemented with 10% serum in a humidified incubator containing 5% CO_2 and 95% air; 1 μCi [5-^{3}H]-dCyd that had previously been reduced to dryness under nitrogen gas was then added and the cells were incubated with 4-S-CAP or N-Ac-4-S-CAP for an additional 90 min. The reaction was terminated by the transfer of 0.5-ml aliquots into 1.5-ml microfuge tubes containing 0.5 ml activated-charcoal suspension (300 mg charcoal in 10% TCA). The mixtures were periodically vortexed for 30 min to remove unreacted [5-^{3}H]-dCyd and were then centrifuged for 6 min at 15,000 rpm. Aliquots of 0.5 ml were pipetted off and the radioactivity was determined. All reactions were done in triplicate.

Macromolecular assay. 4-S-CAP and N-Ac-4-S-CAP were tested for inhibition of DNA synthesis in pigmented melanoma cells. For assessment of inhibition of radiolabel ([^{3}H]-dThd, [5-^{3}H]-dCyd, or [5-^{3}H]-dUrd) incorporation, cells were seeded at 7.5×10^4 cells/well in 24-well flat-bottom microtiter plates containing complete RPMI-1640 media supplemented with 10% fetal calf or calf serum. At 24 h after initial plating cells were incubated at 37° C with selected concentrations of 4-S-CAP or N-Ac-4-S-CAP (2 mM – 100 μM) in complete media supplemented with 10% serum in a humidified incubator containing 5% CO_2 and 95% air. Radiolabel was added after a 2-h drug exposure and was maintained in the presence of drug for an additional 90 min. The drug concentrations causing a 50% reduction in radiolabel incorporation (IC_{50}) were recorded. All determinations were done in triplicate.

MTT viability assay. For assessment of the growth-inhibitory activity of 4-S-CAP and N-Ac-4-S-CAP, cell lines were seeded at 3.5×10^4

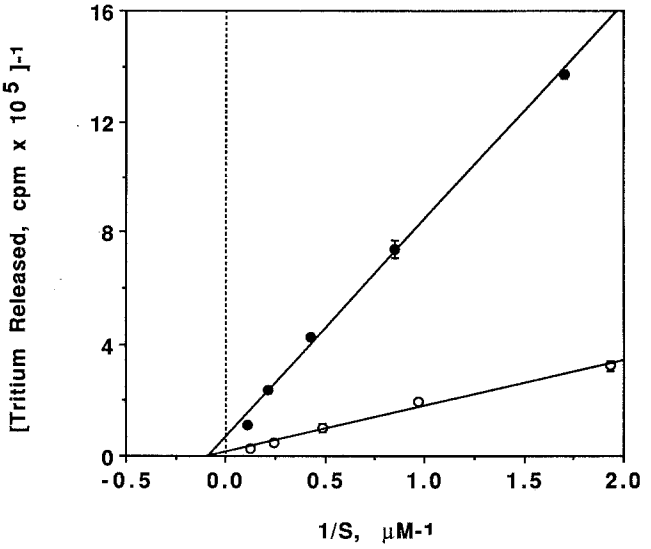

Fig. 1. Dependence of tritium release on the concentration of [5-^{3}H]-dUrd and [5-^{3}H]-dCyd in intact B16-BL6 melanoma cells. Cells were incubated at 37° C for 90 min with selected concentrations of [5-^{3}H]-dUrd (○) or [5-^{3}H]-dCyd (●). The amount of tritiated water released was determined as described in Materials and methods

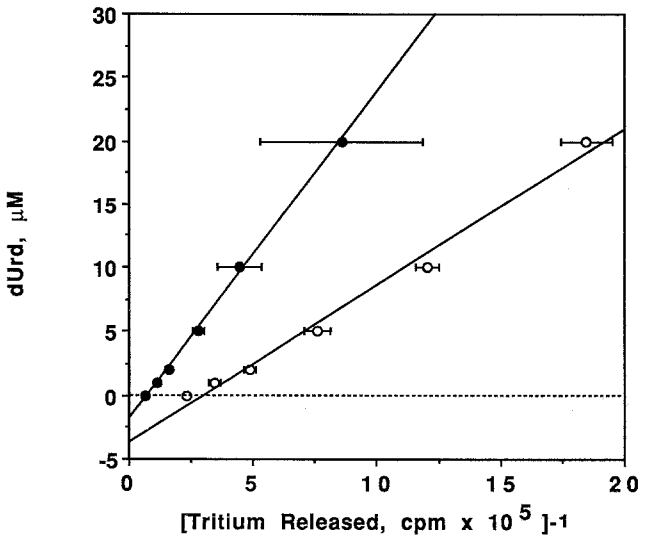

Fig. 2. Isotope dilution plot for thymidylate synthase in intact B16-BL6 melanoma cells. The concentration of unlabeled deoxyuridine was varied with [5-^{3}H]-dUrd (●) and [5-^{3}H]-dCyd (○)

cells/well in 24-well flat-bottom microtiter plates containing complete RPMI-1640 media supplemented with 10% fetal calf or calf serum. At 24 h after initial plating, cell lines were incubated at 37° C with selected concentrations of 4-S-CAP or N-Ac-4-S-CAP (2 mM–10 μM) in complete media supplemented with 10% serum in a humidified incubator containing 5% CO_2 and 95% air. IC_{50} values (drug concentrations causing a 50% reduction in cell viability) were determined by the MTT viability assay after a 48-h incubation period. This assay uses a tetrazolium dye that is reduced to a blue formazan dye by the mitochondria of living cells but not by dead cells or cell debris. The amount of formazan dye reduced is directly proportional to the number of viable target cells remaining [28]. The absorbance of each well was measured on a Fisher Biotech Series 2000 automated microplate reader at an absorbance of 420 nm. All assays were performed in triplicate.

Results

Kinetics of in situ thymidylate synthase activity

Thymidylate synthase-mediated tritium release in situ in the pigmented B16-BL6 melanoma cell line was dependent on the extracellular concentrations of [5-^{3}H]-dUrd and [5-^{3}H]-dCyd (Fig. 1). The linearity of the double-reciprocal plots indicates that the kinetics of the overall process involving radiolabeled nucleoside transport, metabolic formation of [5-^{3}H]-dUMP, and subsequent release of tritium during dTMP synthesis conforms to the Michaelis-Menten equation. The K_m values for [5-^{3}H]-dUrd and [5-^{3}H]-dCyd were 10.8 and 11.8 μM, respectively.

Figure 2 depicts an isotope dilution plot for thymidylate synthase in intact B16-BL6 melanoma cells. The *y*-intercept represents the level of the endogenous pool of compound(s) capable of competing with the labeled precursor for release of tritium by thymidylate synthase [27]. Specifically, the level of the competing substrates was found to be 1.92 μM for [5-^{3}H]-dUrd and 3.68 μM for [5-^{3}H]-dCyd. Rode et al. [26], who have obtained similar values in L1210 leukemia cells, also demonstrated that the rate-limiting step is the reaction catalyzed by thymidylate synthase, thus validating the use of this method for the measurement of in situ thymidylate synthase activity.

Table 1. Comparison of cell lines in situ tyrosinase activity with the inhibition of in situ thymidylate synthase by 4-S-CAP and N-Ac-4-S-CAP

Cell line	Tyrosinase activity[a]	4-S-CAP IC_{50} (μM)[b]	N-Ac-4-S-CAP IC_{50} (μM)[b]
Melanoma:			
B16-BL6	8,441±367	288±41	420±8
B16-F1	7,093±1,264	686±115	588±81
SK-MEL-30	4,385±391	>2,000	520±32
SK-MEL-28	442±118	>2,000	818±42
Nonmelanoma:			
HTB-35	0	>2,000	1,524±61
ZR-75	0	>2,000	>2,000
T-47D	0	>2,000	>2,000

[a] Tyrosinase activity (expressed in counts per minute per 50,000 cells per hour) was determined using the in situ assay described in Materials and methods

[b] Cells were incubated for 2 h with various concentrations of 4-S-CAP and N-Ac-4-S-CAP. They were then exposed to 1 μCi [5-^{3}H]-dCyd for an additional 90 min. IC_{50} values represent the concentration of drug causing a 50% reduction in in situ thymidylate synthase activity

Correlation of in situ tyrosinase activity with inhibition of in situ thymidylate synthase by 4-S-CAP and N-Ac-4-S-CAP

4-S-CAP and N-Ac-4-S-CAP inhibited in situ thymidylate synthase activity to varying degrees in melanoma and nonmelanoma cell lines (Table 1). Only the heavily pigmented B16 cell lines (B16-BL6 and B16-F1) demonstrated inhibi-

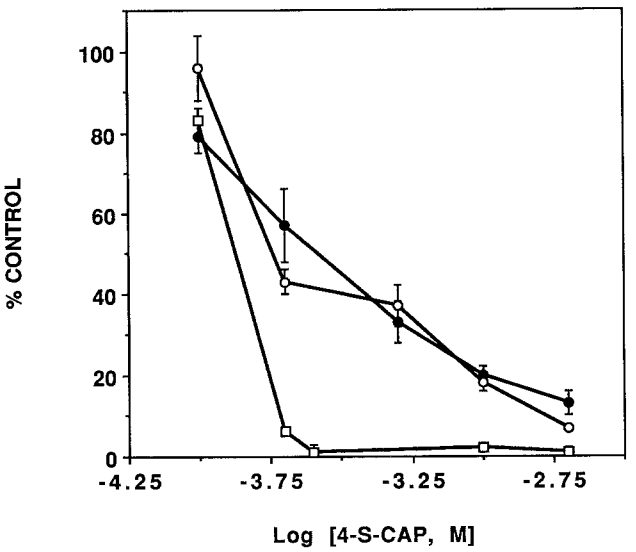

Fig. 3. Correlation of the inhibition of thymidylate synthase (●), DNA synthesis (○), and cell growth (□) by 4-S-CAP in pigmented B16-BL6 melanoma cells. For determination of the effects of 4-S-CAP on DNA synthesis and cell growth, [5-^{3}H]-dCyd incorporation and the MTT viability assay, respectively, were used

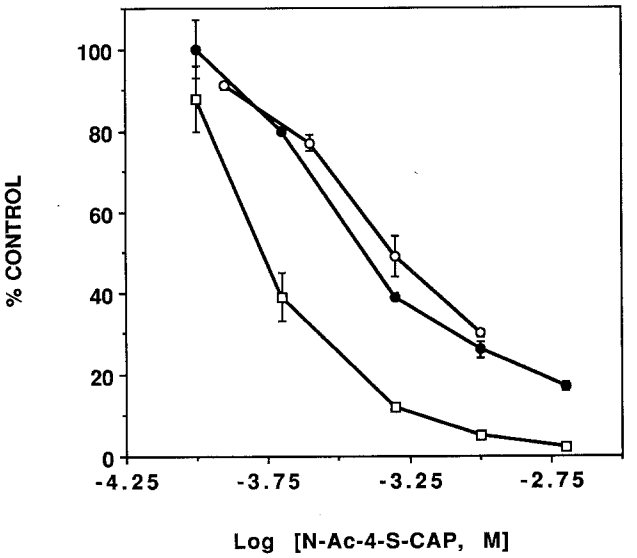

Fig. 4. Correlation of the inhibition of thymidylate synthase (●), DNA synthesis (□), and cell growth (○) by N-Ac-4-S-CAP in pigmented B16-BL6 melanoma cells. For determination of the effects of N-Ac-4-S-CAP on DNA synthesis and cell growth [^{3}H]-dThd incorporation and the MTT viability assay, respectively, were used

tion of in situ thymidylate synthase activity by 4-S-CAP. The IC_{50} values found for B16-BL6 cells were 2.4 times lower than that determined for B16-F1 cells. All melanoma cell lines demonstrated in situ thymidylate synthase inhibition by N-Ac-4-S-CAP. The heavily pigmented B16-BL6 cells were the most sensitive (IC_{50}, 420 ± 8 μM), whereas the lightly pigmented SK-MEL-28 cells were the least sensitive (IC_{50}, 818 ± 42 μM). The IC_{50} value determined for the human HTB-35 squamous-cell carcinoma cell line was 1,524 ± 61 μM, and that found for the human ZR-75 and T-47D breast-carcinoma cell lines was >2 mM for N-Ac-4-S-CAP.

Effect of 5-formyl-tetrahydrofolate on the inhibition of thymidylate synthase by N-Ac-4-S-CAP

Thymidylate synthase catalyzes the final reaction in the de novo synthesis of thymidylic acid (TMP) via the reductive transfer of a single carbon group from N^5, N^{10}-methylene tetrahydrofolate to position 5 of dUMP [9]. Two drugs known to inhibit this enzyme are 5-flourodeoxyuridine (FUDR), which is phosphorylated to fluorodeoxyuridine monophosphate (FdUMP), an inhibitor of thymidylate synthase, and methotrexate, which inhibits the formation of tetrahydrofolate, the reducing substrate for this reaction. Methotrexate cytotoxicity is inhibited by exogenous 5-formyl-tetrahydrofolate (5-F-THF) [19]. To test whether N-Ac-4-S-CAP inhibits thymidylate synthase directly or indirectly by inhibiting dihydrofolate reductase and, thus, the formation of tetrahydrofolate, we subjected cells to incubation with exogenous 5-F-THF. The IC_{50} value determined for inhibition of thymidylate synthase in the presence of 5-F-THF was 553 ± 33 μM, and that obtained in its absence was 425 ± 55 μM. 5-F-THF supplementation nonsignificantly decreased (P >0.005) the IC_{50} value by only 23%, suggesting that inhibition of thymidylate synthase by N-Ac-4-S-CAP is not due to an indirect effect caused by inhibition of dihydrofolate reductase.

Correlation of the inhibition of thymidylate synthase with DNA synthesis and cell growth by 4-S-CAP and N-Ac-4-S-CAP

The inhibition of thymidylate synthase by 4-S-CAP in pigmented B16-BL6 melanoma cells was closely correlated with inhibition of DNA synthesis and cell growth (Fig. 3). The IC_{50} value for in situ thymidylate synthase inhibition by 4-S-CAP was 288 ± 40 μM; the corresponding values for inhibition of cell growth and DNA synthesis were 164 ± 8 and 187 ± 21 μM, respectively. The inhibition of thymidylate synthase by N-Ac-4-S-CAP in the same cells was also correlated with inhibition of DNA synthesis and growth (Fig. 4). The IC_{50} value for inhibition of in situ thymidylate synthase was 420 ± 8 μM; the corresponding values for inhibition of cell growth and DNA synthesis were 491 ± 34 and 216 ± 30 μM, respectively. In nonmelanoma cell lines (e.g., ZR-75 and T-47D), the IC_{50} values for N-Ac-4-S-CAP and 4-S-CAP were >2 mM for the inhibition of both in situ thymidylate synthase activity (Table 1) and cell growth (data not shown).

Effects of N-Ac-4-S-CAP on nucleotide incorporation

N-Ac-4-S-CAP stimulated the incorporation of [5-^{3}H]-dCyd but inhibited [^{3}H]-dThd incorporation in B16-BL6 melanoma cells. Figure 5 shows that [^{3}H]-dThd incorporation was inhibited in a dose-dependent manner at all N-Ac-4-S-CAP concentrations tested. The IC_{50} value for inhibition of [^{3}H]-dThd uptake was 216 ± 30 μM. The op-

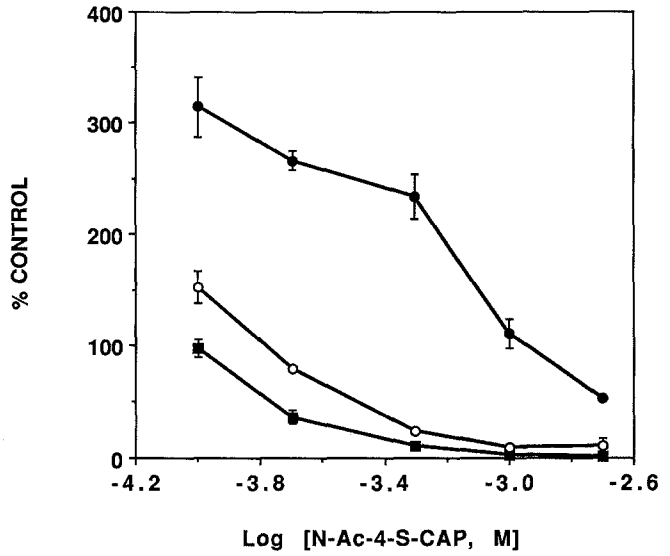

Fig. 5. Effects of N-Ac-4-S-CAP on [5-^{3}H]-dCyd (●), [^{3}H]-dThd (■), and [5-^{3}H]-dUrd (○) incorporation in pigmented B16-BL6 melanoma cells. Cells were exposed to N-Ac-4-S-CAP for 90 min prior to the addition of radiolabel. N-Ac-4-S-CAP was present throughout the period of exposure to radiolabel. After an additional 90-min exposure period, the level of radiolabel incorporation into DNA was determined

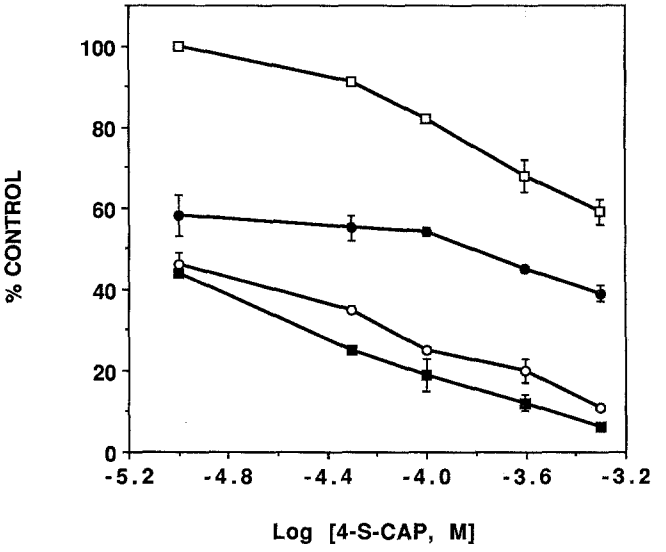

Fig. 6. Inhibition of thymidylate synthase activity by oxidized 4-S-CAP and protection in the presence of reducing agents. Sulfhydryl-containing antioxidants were preincubated with oxidized 4-S-CAP for 15 min prior to the addition of the cell-free lysate containing thymidylate synthase. Inhibition of thymidylate synthase by oxidized 4-S-CAP was determined in the absence of antioxidant (○) and in the presence of 5 mM cysteine (■), 5 mM reduced glutathione (●), or 5 mM dithioerythritol (□)

posite effect was observed for [5-^{3}H]-dCyd incorporation. At a 2-mM drug concentration, a decrease in the incorporation of [5-^{3}H]-dCyd was observed. An increase in [5-^{3}H]-dCyd incorporation occurred at a concentration of 1 mM (i.e., an 11% increase above control values), and this increase continued in a dose-dependent manner as the drug concentration was decreased to 100 μM. At a 100-μM drug concentration, [5-^{3}H]-dCyd uptake was maximally increased by 214% as compared with control values. Unlike N-Ac-4-S-CAP, 4-S-CAP inhibited [5-^{3}H]-dCyd incorporation at all drug concentrations tested. N-Ac-4-S-CAP inhibited [5-^{3}H]-dUrd uptake in the B16-BL6 cell line at all drug concentrations tested except 100 μM, at which a 53% increase in incorporation above control levels was observed.

Reducing agents protect thymidylate synthase activity form oxidized 4-S-CAP

4-S-CAP inhibition of thymidylate synthase activity in cell-free extracts required oxidation of the drug. In the absence of tyrosinase, a concentration of 500 μM 4-S-CAP did not inhibit thymidylate synthase activity (data not shown). Sulfhydryl-containing reducing agents blocked the inhibition of thymidylate synthase activity by oxidized 4-S-CAP to varying degrees (Fig. 6). Antioxidants were incubated for 15 min with oxidized 4-S-CAP prior to the addition of cell-free extract containing thymidylate synthase. The IC_{50} value for the inhibition of thymidylate synthase by oxidized 4-S-CAP or 4-S-CAP plus 5 mM L-cysteine was <10 μM, that for 4-S-CAP plus 5 mM glutathione was 175 ± 3 μM, and that for 4-S-CAP plus 5 mM dithioerythritol was >500 μM. Although dithioerythritol and glutathione significantly blocked (P <0.005) the inhibition of thymidylate synthase by oxidized 4-S-CAP, cysteine had no effect.

Effects of theophylline treatments on N-Ac-4-S-CAP inhibition of thymidylate synthase

Theophylline, a cAMP phosphodiesterase inhibitor, increased tyrosinase activity in B16-BL6 cells and potentiated the inhibition of thymidylate synthase by N-Ac-4-S-CAP. Theophylline (1 mM) treatment significantly increased (P <0.005) in situ tyrosinase activity by 2.3 times after a 24-h incubation period and by 6.3 times after a 48-h incubation period relative to the values obtained in nontreated cells (data not shown). The IC_{50} value found for the inhibition of in situ thymidylate synthase by N-Ac-4-S-CAP in the presence of theophylline after a 24-h exposure period was 281 ± 6 μM, and that determined in its absence was 679 ± 20 μM (Fig. 7). Theophylline significantly decreased (P <0.005) the IC_{50} value for N-Ac-4-S-CAP by 58% in pigmented melanoma cells.

Discussion

In the present study, we measured the release of tritium from [5-^{3}H]-dCyd in intact cells to determine thymidylate synthase activity and to examine the inhibitory effects of 4-S-CAP and N-Ac-4-S-CAP on intact cells. Although the dUrd-to-dTMP pathway is the most direct route, it was not used in our study because thymidine kinase activity varies markedly with the stage of the mitotic cycle and depends more heavily on the proliferation state of the cells than does deoxycytidine kinase [2].

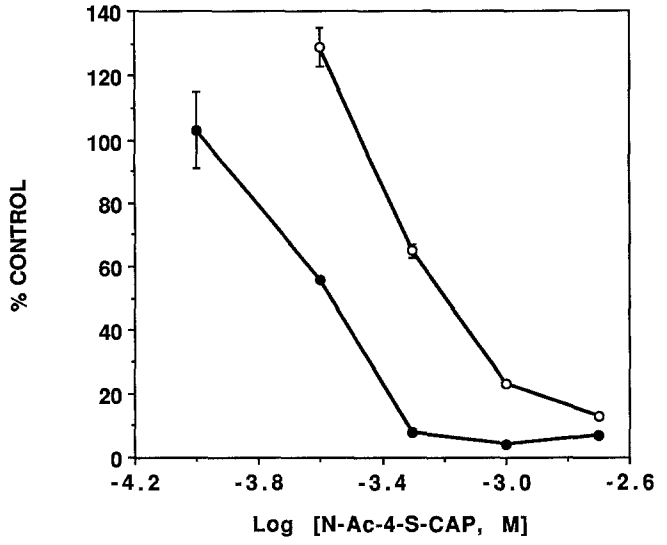

Fig. 7. Theophylline potentiation of the inhibitory activity of N-Ac-4-S-CAP against thymidylate synthase in intact B16-BL6 cells. Cells were preincubated with 1 mM theophylline at 37° C in an atmosphere containing 5% CO_2 for 24 h prior to the addition of N-Ac-4-S-CAP. After an additional 24 h in the presence (●) or absence (○) of theophylline, 1 μCi [5-^{3}H]-dCyd was added for 90 min. The amount of tritiated water released was determined as described in Materials methods

The heavily pigmented melanoma cell lines were most sensitive to the inhibition of in situ thymidylate synthase activity by 4-S-CAP and N-Ac-4-S-CAP. 4-S-CAP inhibited in situ thymidylate synthase activity in the heavily pigmented B16-BL6 and B16-F1 melanoma cell lines but had no effect on lightly pigmented melanoma and non-melanoma cell lines. Theophylline treatment increased both the cellular tyrosinase activity and the inhibitory activity of N-Ac-4-S-CAP on thymidylate synthase in intact pigmented melanoma cells. Thymidylate synthase was not inhibited by 4-S-CAP in cell lysates unless tyrosinase was present. In cells of high oxidative potential, i.e., pigmented melanoma cells, the quinone-mediated mechanism of inhibition of DNA synthesis may be uniquely important in the expression of phenolic amine cytotoxicity. N-Ac-4-S-CAP and 4-S-CAP inhibited in situ thymidylate synthase activity in heavily pigmented melanoma cells, and our results suggest that inhibition of DNA synthesis is correlated with inhibition of thymidylate synthase. DNA polymerase has been proposed as the essential enzyme that is inhibited by oxidized phenolic amines [30]. The primary mechanism of action of drugs that inhibit DNA polymerase leads to elevated levels of deoxynucleoside triphosphate pools, specifically deoxythymidine triphosphate (dTTP). The possibility that elevated dTTP pools feed back and inhibit thymidylate synthase has been ruled out by Reddy [24], who has shown that neither deoxythymidine monophosphate (dTMP) nor dTTP has any effect on thymidylate synthase activity in permeabilized cells. Thus, the primary site of inhibitory activity of these compounds seems to be thymidylate synthase.

Yamada et al. [30] have demonstrated that 4-S-CAP inhibits DNA synthesis in pigmented melanoma cells, whereas RNA synthesis and protein synthesis remain largely unaffected. Since thymidylate synthase represents the sole de novo pathway for dTMP synthesis, a blockade of its activity would have dramatic effects on proliferating cells. Indeed, inhibition of dTMP synthesis leads to a complex situation in which DNA synthesis is impaired but protein synthesis and RNA synthesis are not, a phenomenon termed "thymineless death" [5]. A proposed mechanism of cell death following thymidylate synthase inhibition (i.e. thymineless death) is based on the accumulation of deoxyuridine triphosphate (dUTP), which ultimately leads to DNA damage and cell death [10, 12].

In the present study, N-Ac-4-S-CAP increased the incorporation of [5-^{3}H]-dCyd into DNA in pigmented B16-BL6 cells. Indeed, none of the [5-^{3}H]-dCyd can be detected as radiolabeled dTMP in DNA since the tritium at C-5 of the pyrimidine ring is exchanged for a methyl group in the thymidylate synthase reaction [18]. Balzarini and De Clerq [3] have also found that some thymidine analogs (e.g., dUrd, dThd, 5-vinyl-dUrd, 5-Chloro-dUrd, 5-iodo-dUrd, and 5-ethyl-dUrd) stimulate the incorporation of [5-^{3}H]-dCyd into DNA in L1210 leukemia cells. Unlike N-Ac-4-S-CAP, 4-S-CAP inhibited the incorporation of [5-^{3}H]-dCyd into DNA, suggesting possible differences in the mode(s) of action of these two agents. Some thymidine analogs (e.g. 5-fluoro-dUrd, 5-nitro-dUMP, 5-ethynyl-dUrd, and 5-formyl-dUrd) that exert inhibitory action on thymidylate synthase also inhibit [5-^{3}H]-dCyd incorporation into DNA [3]. Identification of the mechanisms by which compounds that inhibit thymidylate synthase increase or reduce the incorporation of [5-^{3}H]-dCyd into DNA requires further investigation.

Glutathione and dithioerythritol effectively blocked the inhibition of thymidylate synthase activity by oxidized 4-S-CAP in cell-free extracts, but cysteine had no effect. Glutathione metabolism is indirectly involved in melanin biosynthesis as a source of cysteine for pheomelanin synthesis [21], and glutathione may act as a scavenger for radicals produced during melanogenesis [6]. In the present investigation, glutathione blocked the inhibitory effect of oxidized 4-S-CAP on thymidylate synthase whereas cysteine did not, suggesting that glutathione may be essential in maintaining the viability of melanoma cells by scavenging free radicals produced during melanogenesis. Buthionine sulfoximine, an inhibitor of γ-glutaminylcysteine synthetase, the rate-limiting enzyme in glutathione synthesis, is selectively toxic to melanoma cells [17, 23]. Future studies using these and other appropriate model systems are necessary to clarify the relationship between tyrosinase and cellular glutathione levels and to characterize the altered sensitivity of enzymes involved in DNA synthesis to phenolic amine compounds.

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