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Synthesis and Primary Screening for Growth Inhibitors of L1210 Cells of Cholesteryl *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthioacetate

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Abstract □ Cholesteryl *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthioacetate (2) was synthesized:an intermediate, *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthioacetic acid (1) is a congener of an antitumor chlorambucil which both the $-CH_2CH_2-$ linkage and $-N(CH_2CH_2Cl)_2$ group of chlorambucil molecule is doubly modified into the respective -S- linkage and $-NH-CO-NNO-CH_2CH_2Cl$ group. The attackment of cholesterol moiety as a carrier group to *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthioacetic acid was accomplished through the esterification of cholesterol with *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthioacetyl chloride which was obtained from the treatment of *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthioaceticacid with $SOCl_2$. *p*-[3-(2-chloroethyl)ureido]-phenylthioacetic acid was nitrosated with $NaNO_2$ in 98~100% HCOOH to give exclusively *p*-[3-(2-chloroethyl)-3-nitrosoureido]phenylthioacetic acid. Antitumor evaluation of compounds, 1 and 2 on L 1210 leukemia did show significant activity (ED_{40}: 1.14 μg/ml and 8.4 μg/ml, respectively). Further studies were subjected.

Keywords □ Nitrosoureas, Antitumor agent, Lipophilic carrier, Spectral symmetry and asymmetry, Murine leukemic L 1210, CCNU, BCNU, Methyl-CCNU, ED_{50}.

Among significant compounds, nitrosoureas are an extremely active class of antitumor agents that are effective against solid tumors, as well as leukemia. In particular, 2-chloroethyl derivatives and some of their metabolites show great promise as effective antitumor agents[1-3]. For the treatment of a number of experimental and clinical tumors, several N-(2-chloroethyl)-N-nitrosoureas have successfully been applied as chemotherapeutic agents[2]. Not only do these drugs show the ability to inhibit the growth and spread of many forms of solid tumors in men and animals[2,4,5] but some of them, such as N,N′-bis(2-chloroethyl)-N′-nitrosourea (BCNU; NSC-409962) and-(2-chloroethyl) -N′-cyclohexyl-nitrosourea (CCNU, USC-29037), also have been found to rapidly enter the crebrospinal fluid and control menigeal tumor implants.[6] All of these compounds are undergoing intense clinical trials, and some of them have recently been made commerci ally available.

As part of a program aimed at developing antitumor compounds, we have been incorporated an alkylating 2-chloroethyl-nitrosourea group into the chloesterol carrier system in an attempt to prepare compounds which are distributed in such a way that both carrier effect of cholesterol[7] and tumor inhibitory activity of the nitrosourea group can be achieved with the samemolecule[8] Chlorambucil (NSC-3088) has been most commonly used in the chemotherapy of antitumor treatment in man. The simultaneous structural modifications of both the $-N(CH_2CH_2Cl)_2$ group and $-CH_2CH_2-$linkage in the chlora-

mbucil molecule into the respective nitrosourea -NHCO-NNO-CH_2CH_2Cl function and -S- linkage, would give a sulfur-containing congener of chlorambucil of *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthioaceticacid (**1**), and would offer[9] the possibility for additional changes in the potency and selectivity of the simplified compound **1**, possibly in its antitumor inhibitory effectiveness. Since the lipophilic steroid moieties played an inportant role to act as a specific carrier to transport the compound into tumor cells[10], the study is extended to synthesize cholesteryl *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthio acetate(**2**).

EXPERIMENTAL METHODS

Melting points with a range were determined by polytemp (Polyscience Corporation) and Fisher-Johns apparatus and are uncorrected.^{1}H NMR spectra were recorded on a Varian EM-360 spectrometer in DMSO-d_6 with $(CH_3)_4Si$ as an internal standard, and IR spectra with Shimadzu 400 spectrophotometer. Thin layer chromatography plates (3×9cm) were made with slurry medium of 30g of Silica Gel G Type 60 and 100ml of $CHCl_3 \cdot CH_3OH$ (2:1, v/v) and their chromatograms were developed with a methanol solvent and detected by iodine vapor. N,N-Dimethylformamide was fractionally distilled under reduced pressure and anhydrous conditions after drying over KOH pellets. Ater discarding the forerun, the major fraction distilling within a range of 4°C was collected and stored over 4°A molecular sieves and under N_2 in a tightly sealed bottle and kept under refrigeration. Prior to use, DMF was tested for the pressnce of dimethylamine with 3,4-dinitrofluorobenzene. *p*-Amino phenylthioacetic acid and 2-chloroethyl isocyanate were products of the respective Reiley Chemical Co. and Eastman Kodak Co.

p-[3-(2-Chloroethyl)ureido]phenylthioacetic Acid(**4**)

To a 50ml of round-bottomed flask contained 0.55g (3mmole) of P-amino phenylthioacetic acid in 12ml of chloroform was added 0.42ml (3m mole) of triethylamine, and the reaction mixture was stirred at room temperature for 30 min. To a stirred reaction mixture was added slowly 0.26ml (3m mole) of 2-chloroethyl isocyanate and mixture was continously stirred for 6 hours at room temperature. On the end of the reaction (The end of the reaction was checked by the TLC), the mixture was filtered and the filtrate was extracted with three portions of 15ml of distilled water. The combined water extractions (pH=6) was made with 1ml of conc. HCl acidic, and found white solids. The white solids were triturated with water several times, and crystallizde with ethanol-water to afford 0.61g (84%) after dried on 60 mm/Hg at 60°C, MP 141-142°C. IR (KBr) showed an appearance of a new urea peak at 1725cm^{-1}. Rf=0.75. NMR (DMSO-d_6), δ 3.33-3.73 (m, $-SCH_2-$, $ClCH_2CH_2-$), 6,43 (m, $Cl-CH_2CH_2-NH-$), 8.74 (s, aromatic-NH) and 2.5 (m, residual DMSO-d_6).

p-[3-(2-Chloroethyl)-3-nitrosoureiod]phenylthio acetic Acid (**1**)

To a solution of 0.49g (1.7m mole) of **4** in 98-100% HCOOH (15ml) was cooled to 0-5°C and treated with 0.504g (7.3m mole) of $NaNO_2$ in several portions. The reaction mixture was stired for 45 min at 0-5°C and found yellow solids. The whole reaction mixture was diluted with ice-cold distiled water (70ml) and filtered. The yellew solids were triturated with water several times until the washings were pH=7. Crystallization from ethanol-water gave 0.41g

(76%) of yellow solids. MP 93-94°C. R*f*=0.81, IR (KBr), 1721cm^{-1} (urea carbonyl peak due to nitroso inductive effect), and 1482cm^{-1} (nitroso peak). NMR (DMSO-d_6), δ 3.55 and 4.25 (t, 4H, -NNO-CH_2CH_2Cl), 10.85 (s, 1H, Aromatic-NH-). λ_{Max}^{EtOH} 398 nm (ε=337) and 415 nm (ε=270)[13].

Elemental Anal.: Obs. (Calc.%) C, 43.37 (43.79); H, 4.19 (4.01), N, 13.57 (13.93).

Choresteryl p-[3-(2-chloroethyl)-3-nitrsoureido] phenylthioacetate(2)

A mixture of 0.16g (0.5m mole) of **1** and a freshly distilled $SOCl_2$ (8ml) was refluxed for 1 h and the excess $SOCl_2$ was evaporated in vaccuo to afford oily residues. The oily residues were dissolved in anhydrous pyridine(5ml) and added dropwise to the solution of 0.14g (0.35 m mole) of cholesterol in 5ml of pyridine solution. The reaction mixture was stirred at room temperature for 20h, and the mixture was poured into the 50ml of ice-water. The yellow solids were washed with ice-cold distilled water several times until the washings were neutral. Crystallization form chloroform-cyclohexane gave 0.25g (93%) of yellow solid. MP 160.5-162°C. R*f*=0.62. IR (KBr), 1722cm^{-1}(Amidel band), 1740cm^{-1} (ester carbonyl) and 1482 cm^{-1} (Nitroso Peak). NMR (DMSO-d_6), δ 3.56 (t-CH_2Cl), 4.25 (t,-NNO-CH_2-), 10.85 (s, Aromat-NH) 0.5-2.2 (m, steroid-H). λ_{max}^{EOH} 510nm (ε=652). Elemental Anal.; Obs. (Calc. %) C. 69.47 (69.74); H, 8.60 (8.63); N,6.31 (6.42).

Cholesteryl p-[3-(2-chloroethyl)ureido]phenylthioacetate(8)

A mixture of 0.29g (1m mole) of **4** in a freshly distilled acetonitrile (80 ml) and 0.8ml (10m mole) of $SOCl_2$ was refluxed for 24h and the excess $SOCl_2$ was evaporated in vaccuo to afford oily residues. The oily residues were dissolved in 15ml of pyridine and added dropwise to the solution of 0.32g (0.8m mole) of cholesterol in 5ml of pyridine solution. The reaction mixture was stirred at room temperature for 24h and the mixture was poured into the 30ml of ice-cold water. The yellow solids obtained, were crystallized from acetone (active charcoal) to afford yellow solids (0.45g, 80%). MP 137-138°C. R*f*=0.79. IR (KBr), 1739cm^{-1} (ester peak), 1720cm^{-1} (Amide I band). NMR (DMSO-d_6), δ 3.37-3.79 (m, SCH_2-, $ClCH_2CH_2$-), 6.44 (m, $ClCH_2CH_2$-NH), 8.77 (s, Aromatic-NH). 0.5-2.37 (m,Steroid-H). Elemental Anal.; Obs (Calc. %), 46.47 (46.23); H, 419 (4.23); N, 1457 (14.71).

Antitumor Evaluation

Cancer Cell

Murine leukemic lymphoblast, L 1210, was obtained from Perman's laboratory, University of Wisconsin. The cells are round and grow by obinary division. They have doubling time of 12 to 18 hours under favorable conditions. The culture has been maintained by growing in screw-capped tubes (20×150mm) at 37°C and transferring twice a week.

Culture Medium

Fischer's powder medium for leukemic cells of mice (H-II) was purchased from Gibco Laboratories, Grand Island, New York, USA. Horse serum was supplied locally by taking fresh blood immediately after the slaughter directly in 500ml centrifuge tubes. The tubes were stood at room temperature for a couple of hours. After the blood clotted, the tubes were centrifuged for 10 minutes at 5000 rpm. The supernatants were sterilized using Milipore filters and stored at-20°C until use.

To make one liter of the culture medium, a package of Fischer's powder medium (1-liter package) was dissolved in redistilled water. Added 1.125 grams of $NaHCO_3$ and 100ml of

hourse serum. Adjusted the pH to 7.2. Filter sterilized and stored in the refrigerator. Just before use added 100 units/ml of penicillin and 0.1 mg/ml of streptomycin.

Chemicals

A positive control compound, methyl-CCNU (NSC 95441, 1-(2-chloroethyl)-3-(trans-4-methylcyclothexyl)-1-nitrosourea) was obtained from NCI (National Cancer Institute). All other chemicals were of analytical grade.

Culture Preparation

Spinner cultures (cells in logarithmic phase of growth) were prepared in 250-ml screw-capped Ehrlen meyor flask. The L 1210 cells were inoculated to a 50-ml of prewarmed medium to make an initial concentration of 2 to 5×10^5 cells per ml a day prior to the assay. Through an incubation at 37°C the cell concentration usually reached to 0.8 to 1.0×10^6 cells per ml in 24 hours.

The spinner cultures were diluted with prewarmed fresh medium to make final concentration of 5.5×10^4 cells per ml immediately before the cells were dispensed into the individual growth tubes. This culture suspension is called "run bottle" and used to distribute 5ml each in screw-capped culture tubes.

ED_{50} Determination

Exactly 0.5ml each of the compounds **1** & **2** (made 0.5 mg/0.5 ml) was added to the culture tubes having 5ml each of "run bottle". The same volume of distilled water was added instead of compounds **1** and **2** for control tests. For the positive control tests, different concentrations of methyl CCNU, a known cytotoxic compound, was used instead of compounds **1** and **2**. In all cases the total liquid volume per tube was 5.5ml having final cell concentration around 5×10^4 cells per ml. For each dose duplicate tubes were prepared.

The culture tubes thus prepared were incubated stationally at 37°C for 48 hours. Cell numbers were then counted using a coulter electronic particle counter (Electrozone Cellosc-ope, Particle Data, Inc., Elmhurst, III. USA) standardized by hemocytometerr Duplicate counts for each tube were carried, and the mean of four values at one dose level was used for growth ratio determinations.

RESULTS AND DISCUSSION

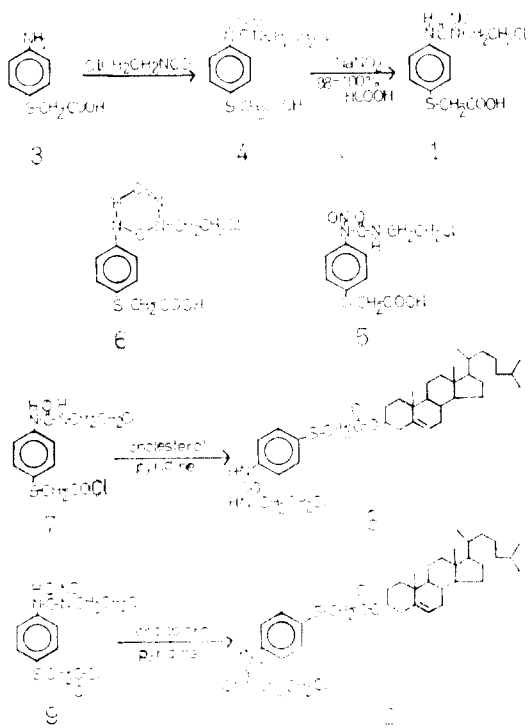
Chemicals

For the synthesis of cholesteryl *p*-[3-(2-chloroethyl)-3-nitrosoureido]phenylthioacetate (**2**), the key intermediate, *p*-[3-(2-chloroethyl)-3-nitrosoureido]phenylthioaceticacid (**1**) was prepared by a series of conversions starting from P-amino phenylthio acetic acid (**3**)[11]. The compound **3** easily reacted with 2-chloroethyl isocyanate to give moderate yields of *p*-[3-(2-chloroethyl)ureido]phenylthioaceticacid (**4**). The infrared spectrum of **4** shows a new urea absorption peak at 1625 cm^{-1}, The nuclear magnetic resonance spectrum of **4** indicates the spectral asymmetry of the Cl-CH_2-CH_2-NH-group (A_2B_2X system) which showed the multiplicity (δ 3.33-3.73) of the methylene groups due to the -NH coupling with the adjacent methylene group. The complete deuterium exchange (DMSO d_6-D_2O) of **4** resulted in the disappearance of -NH signals (δ 6.43 of Cl-CH_2CH_2-NH, and δ 8.73 of aromatic-NH), and appeared as a complete spectral symmetry (A_2B_2 system) of Cl-CH_2-CH_2-ND-, showing triplet of the methylene groups at δ 3.39 and 3.38.

Nitrosation of **4** with 98~100% HCOOH and dry $NaNO_2$ would be expected to yield predominantly the nitrosourea **1**. The isomeric purity of a nitrosourea **1** was established by nuclear magnetic resonance spectroscopy. The spectral

asymmetry of the -NNO-CO-NH_2CH_2-Cl (A_2B_2 X system) group (isomer 5) due to the NH coupling of the adjacent methylene group can be clearly distinguished from the spectral symmetry of -NH-CO-NNO-CH_2CH_2-Cl (A_2B_2 system) group (isomer 1). The presence of two distinct triplets (A_2B_2 system) centered at δ 3.55 and 4.25 in the nuclear magnetic resonance spectrum was strong evidence that the nitroso group was attached to the same nitrogen as the chloroethyl group (-NNO-CH_2CH_2-Cl).

The extreme downfield shift (δ 10.85) of the -NH proton (Ar-NH-CO-NNO-) in compound 1 led to the supposition that the -NH proton was strongly hydrogen-bonded with the strong electronegative nitroso group as shown in 6.



The D_2O exchange of 1 showed no absorption signal at δ 10.85. The infrared spectrum of 1 showed a band at 1482cm^{-1}, indicating the presence of a nitroso group. Furthermore, the sharp absorption at 1721cm^{-1} was characteristic of the shift to a higher wavenumber of the urea carbonyl absorption caused by the strong inductive effect of nitroso group of the ureido funtion.[12] The ultraviolet spectrum of 1 showed two maxima[13] at 398 (ε=337) and 415 nm (ε=270), in contrast to the urea derivative 5 (310 nm, ε=275).

It was reasoned that an appropriate synthetic route for 2 could be that the compound 4 was reacted with $SOCl_2$ to give *p*-[3-(2-chloroethyl) -ureido] phenylthio acetyl chloride (7) which was esterified under pyridine solvent to afford cholesteryl *p*-[3-(2-chloroethyl)ureido]phenylthioacetate (8) in 80% yields. Attempts to nitrosate 8 with $NaNO_2$ in 99% HCOOH led to intractable tars and the difficulties in freeing the homogeneous material from the reaction products.The direct esterification of cholesterol with the compound 1 under Dean-Stark water trap was also failed and the *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthio acetyl chloride (9) obtained from the treatment of 1 with $SOCl_2$, was subjected to esterify with cholesterol to afford yellow crystals, cholesteryl *p*-[3-(2-chloroethyl)-3-nitrosoureido] phenylthio acetate (2) in good yields. The infrared spectrum of 2 showed an appearance of ester peak at 1740 cm^{-1} and the result of elemental analysis corresponds to the correct molecular formular.

Biological

In the present study murine leukemic L 1210 cells were used and no criterion for this type of cells is available. As an alternative way a positive control test with a known anticancer agent, methyl-CCNU, was performed. Studies on the mechanism of BCNU action had shown alkylating activity in DNA of the cell[14] and CCNU and methyl-CCNU might have similar activity in DNA. Because of the superior exper-

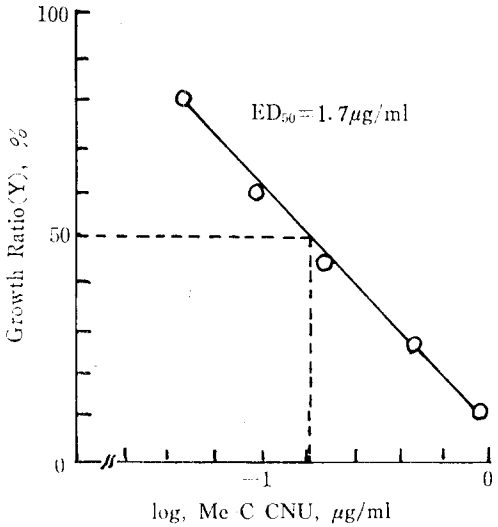


Fig. 1: ED_{50} value of methyl-CCNU on L1210 cells.

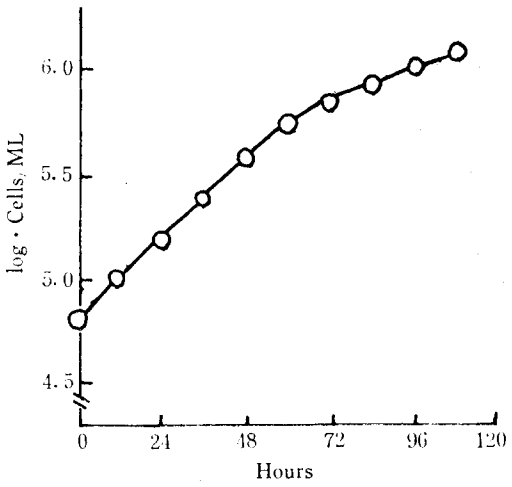


Fig. 2: Growth of L1210 cells in Fischer's medium fortified with horse serum and sodium bicarbonate The cells were grown stationary in screw-capped tubes at 37°C.

mental activity in leukemic cell and great lipid solubility than BCNU, methyl-CCNU was selected for clinical trials. Under the NCI protocol, the quality control limit has been tentatively set at a ED_{50} value range of 1.7 to 7.7 μg per ml. When the same compound, methyl-CCNU, was applied to the present testing cells of L1210, the ED_{50} value was 1.7 μg per ml (Fig. 1) which laid in the lower end of the NCI quality control limit range.

Growth of L1210 Cells in Fischer' s Medium

Fig. 2 shows a typical growth pattern of L 1210 cells in the Fischer's medium without any inhibiting substance added. When the cell concentrations were plotted semilogarithmically against culture time, a straight line could be observed at least for 48 hours which was used as culture period in this study. The doubling time of L1210 cells during the logarithmic growth period was computed as 15.6 hours.

Positive Control Test

Following the NCI manual[15] the ED_{50} value of a known anticancer agent, methyl-CCNU, was determined to check reliability of our procedures. The growth ratio for each dose of testing substance, Y, was calculated by the following

$$\frac{T-Co}{C-Co} \times 100 = Y(\%)$$

where T= mean cell count for each dose of testing substance after 48 hours incubation; C= mean cell count for control after 48 hours incubation, Co=mean cell count at the start of incubation.

When Y values were plotted against doses of methyl-CCNU semi-logarithmically, a straight line could be obtained as shown in Fig. 1. Using the straight line a concentration of methyl-CCNU which could inhibit the growth of L1210 cells by 50% (ED_{50}) was estimated as 1.7 μg per ml.

Growth Inhibitors of Compounds **1** *and* **2**

The value of ED_{50} which is the synthesized compounds which inhibit the growth of cells to the level of 50% of the untreated control was then determined by the procedure described in

the NCI manual.[15] Under the NCI protocol, the synthesized compounds **1** and **2** showed significantly low ED_{50} values of 1.14 μg/ml and 8.4 μg/ml, respectively. Further studies were subjected and the results will be published in later date.

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