Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXIX, No. 2

SYNTHESIS OF HIGH SPECIFIC ACTIVITY TRITIUM-LABELLED CHLOROETHYLCYCLOHEXYLNITROSOUREA AND ITS APPLICATION TO THE STUDY OF DNA MODIFICATION

Ernest L. Siew[1], Yvette Habraken[2] and David B. Ludlum[2,3]

[1]Chemistry Department, University at Albany, State University of New York, Albany, NY 12222; and

[2]Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655, U.S.A.

SUMMARY

A small-scale synthesis of high specific activity, N-(2-chloro-2-[^{3}H]-ethyl)-N'-cyclohexyl-N-nitrosourea ([^{3}H]-CCNU) has been accomplished from tritium-labelled ethanolamine. The product is pure by TLC and HPLC analysis and has been used successfully to modify DNA. The overall yield on radioactivity including losses in HPLC purification is approximately 4 percent. The availability of this tritium-labelled compound makes studies of DNA repair and of cellular resistance to N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea possible.

Key Words: Tritium-labelled 2-haloethylnitrosourea; CCNU

INTRODUCTION

Recent studies indicate that the 2-haloethylnitrosoureas, as well as several other effective antitumor agents, produce their cytotoxic actions by modifying DNA (1-4). However, such agents introduce a variety of modifications into the DNA structure and it is usually not clear which of these are cytotoxic.

One method of investigating this question is to compare the distribution of DNA modifications produced by the agent in a cell line which is sensitive to its action to the distribution in a resistant cell line (5). Since a limited amount of DNA is available from cultured cells, these experiments are feasible only if the agent has a high specific activity. In exploratory studies, this synthesis must often be done on a small scale which introduces additional technical difficulties. Here we report the successful model synthesis of high

---

[3]To whom correspondence should be addressed.

0362-4803/91/020223-08$05.00
© 1991 by John Wiley & Sons, Ltd.

Received September 6, 1990
Revised September 25, 1990

specific activity N-(2-chloro-2-[^{3}H]-ethyl)-N'-cyclohexyl-N-nitrosourea ([^{3}H]-CCNU) using a route developed by Dr. John A. Kepler, Research Triangle Park, NC, for the synthesis of [^{14}C]-CCNU. This [^{3}H]-CCNU has been used to modify DNA, yielding a profile of DNA modifications similar to that produced by [^{14}C]-CCNU.

RESULTS AND DISCUSSION

The synthesis of [^{3}H]-CCNU is shown in Fig. 1; it was performed on a 2.5 mCi scale, or 208 nmol since the specific activity was 12 Ci/mmol. Losses were kept low by limiting the number of transfers and by using a small-scale HPLC unit in the purification steps.

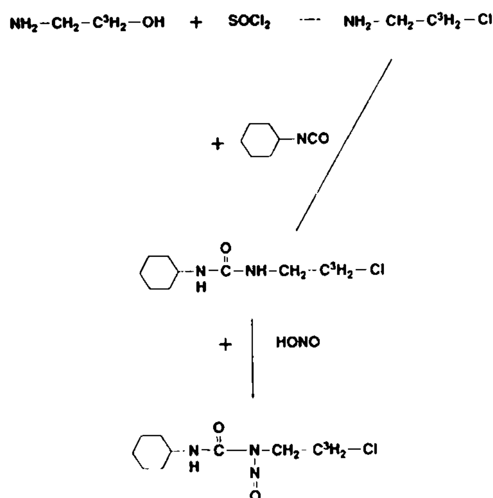

Fig. 1. Synthesis of high specific activity [^{3}H]-CCNU.

1-[^{3}H]-ethan-1-ol-2-amine was converted to 1-[^{3}H]-ethan-1-chloro-2-amine in a 60% yield by treatment with $SOCl_2$ in dichloroethane. Volatiles were removed, and 1-[^{3}H]-ethan-1-chloro-2-amine was condensed with cyclohexyl isocyanate in methylene chloride. The reaction mixture was then partitioned between 0.1 N HCl and $CHCl_3$, and the organic layer was recovered. After TLC verification that chloroethyl cyclohexyl urea (CCU) had been formed, the compound was purified by preparative HPLC as described below. The TLC systems used to document reaction at each step are shown in Table 1.

Table 1. TLC retention times relative to solvent front

Compound	Solvent 1[a]	Solvent 2[b]	Solvent 3[c]
Ethanolamine	0.20	-	-
Chloroethylamine	0.45	0.10	-
CCU	-	0.30	0.0
CCNU	-	-	0.69

[a]Solvent 1: n-butanol/acetic acid/water, 4/1/5, v/v/v
[b]Solvent 2: cyclohexane/acetone, 7/3, v/v
[c]Solvent 3: methylene chloride

HPLC-purified N-(2-chloro-2-[^{3}H]-ethyl)-N'-cyclohexylurea was then nitrosated to give the desired N-(2-chloro-2-[^{3}H]-ethyl)-N'-cyclohexyl-N-nitrosourea. As described in the original synthesis of the unlabelled compound (6) and a more recent synthesis of [^{11}C]-labelled material (7), use of concentrated formic acid and solid sodium nitrite causes the nitrosation reaction to occur primarily at the N rather than the N' nitrogen. N-(2-chloro-2-[^{3}H]-ethyl)-N'-cyclohexyl-N-nitrosourea was recovered and purified by HPLC. Yields in each step including purification steps are given in Table 2.

As a consequence of the small scale in which this reaction was run, it was not possible to obtain any spectroscopic information on the purity of the product. However, Fig. 2 shows the results of co-chromatography of [^{14}C]-CCNU and [^{3}H]-CCNU on an analytical HPLC column. [^{14}C]-CCNU and [^{3}H]-CCNU eluted together as a single peak.

Table 2. Yields at each step in the reaction

Compound	Amount (mCi)	Yield (Percent)
[^{3}H]-Ethanolamine	2.5	--
[^{3}H]-Chloroethylamine	1.5	60
[^{3}H]-CCU, impure	0.69	46
[^{3}H]-CCU, column purified	0.20	29
[^{3}H]-CCNU, impure	0.13	65
[^{3}H]-CCNU, column purified	0.10	77

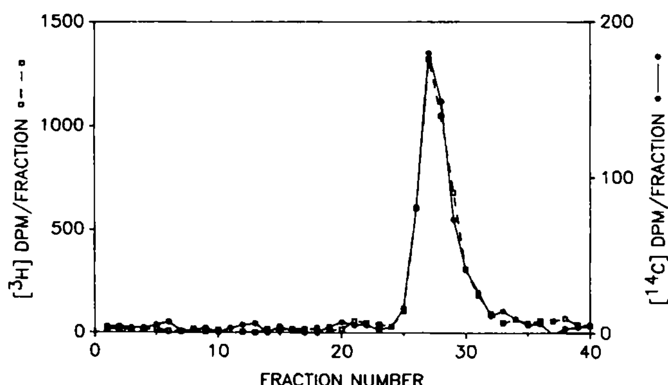

Fig. 2. Co-chromatography of laboratory-synthesized high specific activity [^{3}H]-CCNU and [^{14}C]-CCNU obtained from the National Cancer Institute.

Next, we examined the reaction of [^{3}H]-CCNU with DNA. Purines were released from modified DNA by acid hydrolysis and separated on a C_{18} column, as shown in Fig. 3; purines released from DNA modified with [^{14}C]-CCNU are shown for comparison. Both profiles contained the products, 2-chloroethyl guanine and 2-hydroxyethyl guanine, which were identified by co-chromatography with known markers; other peaks also seem to be represented in both profiles.

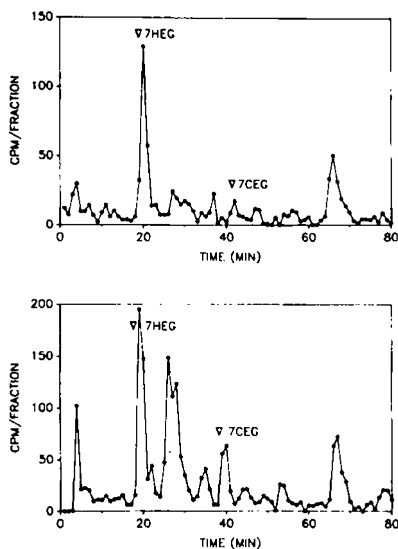

Fig. 3. HPLC profiles of modified bases released from DNA treated with [^{3}H]-CCNU (upper figure) or with [^{14}C]-CCNU (lower figure).

We conclude from this study that it is feasible to synthesize high specific activity DNA-modifying agents on a small scale. These agents can then be used to examine the distribution of DNA modifications in tumor cell lines or to prepare DNA substrates for the investigation of DNA repair.

EXPERIMENTAL

Materials. 1-[^{3}H]-ethan-1-ol-2-amine hydrochloride, 12 Ci/mmol, was obtained from Amersham (Arlington Heights, IL). This compound was prepared by reduction of glycine ethyl ester hydrochloride with sodium borotritide, and the exchangeable tritium was removed by repeated evaporation from a hydroxylic solvent. The compound was purified by paper chromatography and its radiochemical purity was shown to be 98% on TLC analysis in three separate chromatographic systems. N-(2-chloroethyl-U-[^{14}C])-N'-cyclohexyl-N-nitrosourea ([^{14}C]-CCNU), synthesized by Dr. John A. Kepler, Research Triangle Park, NC, was obtained through the National Cancer Institute. HPLC grade solvents were used throughout and were stored over 4Å molecular sieves to remove traces of water. Unlabelled CCNU was obtained from the National Cancer Institute, Division of Cancer Treatment, and N-chloroethyl-N'-cyclohexylurea was prepared as described (6). Other chemicals were reagent grade materials.

Chromatography. Each step in the synthesis was monitored by TLC on silica gel plates precoated on plastic (Macherey-Nagel, Düren, FRG) and cut to 2.5 x 10 cm. TLC solvent 1 was n-butanol/acetic acid/water, 4/1/5, v/v/v; TLC solvent 2, cyclohexane/acetone, 7/3, v/v; and TLC solvent 3, methylene chloride. Unlabelled amines were developed with ninhydrin spray and other markers were visualized with Rhodamine dye. Chromatograms of radiolabelled compounds were cut into 0.75 cm strips and radioactivity was determined in a scintillation counter as described below.

Preparative HPLC separations were performed on a silica gel column (1.6 x 70 mm) which was repacked with 28-200 mesh silica gel, grade 12 (Fisher Scientific Co., Fair Lawn, NJ) after each separation. Silica gel was activated for 16 hr at 110° before use. For purification of [^{3}H]-CCU, columns were eluted at 1 ml/min with cyclohexane/acetone, 85/15, v/v. For purification of [^{3}H]-CCNU, columns were eluted at 0.25 ml/min with methylene chloride. In each case, 1 ml fractions were collected and aliquots were counted as described below. TLC

analysis was performed on each fraction containing radioactivity.

[^{3}H]-CCNU was analyzed on a Spheri-10 10μ silica gel column (4.6 x 100mm) obtained from Brownlee Labs (Santa Clara, CA). [^{14}C]-CCNU was added as a marker and the column was eluted with methylene chloride at 0.3 ml/min; 0.5 min fractions were collected and counted in two channels in a scintillation counter so that radioactivity associated with [^{14}C] and [^{3}H] could be calculated separately.

Liquid scintillation counting. Aliquots of aqueous solutions or strips cut from TLC plates were added to 4.5 ml of Hydrofluor scintillation fluid (National Diagnostics, Manville, NJ) and counted in a Beckman LS 100 scintillation counter in two channels chosen to allow calculation of [^{14}C] and [^{3}H] radioactivity separately. Constants for calculating dpms for the two separate radiolabels and for calculating quench corrections were determined using standard [^{14}C]-hexadecane from Amersham (Arlington Heights, IL) and [^{3}H]-water from New England Nuclear (Boston, MA).

Synthesis. 2.5 mCi of 1-[^{3}H]-ethan-1-ol-2-amine hydrochloride, which was received dissolved in water, was transferred to a 100 mm pyrex test tube with a Teflon-lined screw cap. Water was removed by lyophilization and 1 ml of dichloroethane was added as solvent. Thionyl chloride, 400 μl, was then added and the mixture was incubated at 55° with magnetic stirring. After 5 h, the volatile materials were removed with a stream of nitrogen and the residue was dried under vacuum.

This product was dissolved in 1 ml of methylene chloride, and TLC analysis was performed to verify that chlorination had occurred. Excess acid was neutralized with 80 μl of a 1/200 solution of triethylamine in methylene chloride and 100 μl of cyclohexyl isocyanate was added. The reaction was continued for 90 min at room temperature and the mixture was partitioned between 1 ml of 0.1 N HCl and 1 ml $CHCl_3$. The organic layer was transferred to another pyrex test tube and evaporated to dryness.

After TLC verification that chloroethyl cyclohexyl urea had been formed, purification was achieved by passing the solution, 500 μl at a time, through a preparative HPLC column eluted with cyclohexane/acetone, 85/15, v/v. The first 5 fractions contained pure chloroethyl cyclohexyl urea, as determined by TLC analysis, and were pooled together. Solvent was removed with a gentle stream

of nitrogen and 1.5 ml of 90% formic acid was added. The mixture was cooled in an ice bath and 5 mg of sodium nitrite was added slowly over a 15 min period. Stirring was continued for an additional 30 min and then the mixture was partitioned between 2 ml of water and 2 ml of $CHCl_3$. The organic layer was removed and the $CHCl_3$ was evaporated. The product was dissolved in 1 ml of methylene chloride and the formation of CCNU was verified by TLC analysis. The crude [^{3}H]-CCNU was then purified 400 μl at a time by HPLC as described above. Pure [^{3}H]-CCNU appeared in the first 2 fractions which were pooled together. As shown in Table 2, 100 μCi of material which appeared as a single spot on TLC were obtained for an overall yield of 4%. Each step in the reaction gave a 50 to 60% yield in repeated syntheses, but significant yield losses were also experienced in column purification.

DNA Modification. In order to determine the distribution of modified bases in DNA reacted with radiolabelled CCNU, calf thymus DNA was dissolved at 10 mg/ml in 50 mM sodium cacodylate buffer, pH 7.5. One volume of ethanol was mixed in slowly, and the solution was allowed to rehomogenize for approximately two hours. Then radiolabelled CCNU, which had been dissolved in a small volume of alcohol, was added and the reaction mixture was incubated for 16 h at 37°. Finally, non-covalently bound radioactivity was removed by repeated cycles of ethanol precipitation and redissolution in cacodylate buffer.

Alkylated DNAs were depurinated in 0.1 N HCl for 1 h at 100° and the solution was centrifuged to remove the residual DNA. The supernatant was adjusted to pH 4.5, applied to a DEAE A-25 column (1 ml bed volume) to remove oligodeoxynucleotides, and eluted with 3 ml of water. Modified bases were separated on an Spherisorb 5 μm C_{18} column obtained from Alltech (Deerfield, IL). The column was eluted at 1 ml/min with 25 mM KH_2PO_4, pH 6, for 18.5 min followed by increasing concentrations of acetonitrile in the same buffer: a 0%-5% gradient of acetonitrile over 10 min, 5% acetonitrile for 25 min, a 5%-50% gradient of acetonitrile over 5 min, and 50% acetonitrile for 10 min. One min fractions were collected and radioactivity was determined in a scintillation counter. Optical markers of 7-(2-chloroethyl)guanine and 7-(2-hydroxyethyl)guanine were prepared as described previously (2) and added to the samples before chromatography.

ACKNOWLEDGMENT

This work was supported by Public Health Service Grant CA44499 from the National Cancer Institute. The skilled secretarial assistance of Marion Bonin is gratefully acknowledged.

REFERENCES

1. Ludlum D.B. and Tong W.P. - In: *Nitrosoureas in Cancer Treatment* (Eds. Serrou B., Schein P. and Imbach J.-L.), pp. 21-31. Elsevier/North Holland Biomedical Press, Inc., Amsterdam (1981)
2. Ludlum D.B. and Tong W.P. - In: *Experimental and Clinical Progress in Cancer Chemotherapy* (Ed. Muggia F.M.) pp. 141-154. Martinus Nijhoff, Boston (1985)
3. Kohn K.W., Erickson L.C., Laurent G., Ducore J., Sharkey N. and Ewig R.A. - In: *Nitrosoureas: Current Status and New Developments* (Eds. Prestayko A.W., Crooke S.T., Baker L.H., Carter S.K. and Schein P.S.), pp. 69-83. Academic Press, Inc., New York (1981)
4 Hemminki K. and Ludlum D.B. - J. Natl. Cancer Inst. 73: 1021 (1984)
5. Bodell W.J., Tokuda K. and Ludlum D.B. - Cancer Res. 48: 4489 (1988)
6. Johnson T.P., McCaleb G.S., Oplinger P.A. and Montgomery J.A. - J. Med. Chem. 9: 892 (1966)
7. Diksic M., Farrokhzad S., Yamamoto Y.L. and Feindel, W. - J. Radioanal. Nucl. Chem. 89: 45 (1985)