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3-DEAZANEPLANOCIN A: A NEW INHIBITOR OF *S*-ADENOSYLHOMOCYSTEINE SYNTHESIS AND ITS EFFECTS IN HUMAN COLON CARCINOMA CELLS

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Abstract—The mechanism of action of the cyclopentenyl analogue of 3-deazaadenosine (3-deazaneplanocin A or c^{3}Nep) was investigated in the human colon carcinoma cell line HT-29. Upon exposure of cells for 24 hr to 3-deazaneplanoxin A (c^{3}Nep), neplanocin A (Nep) or 3-deazaaristeromycin (c^{3}Ari), significant toxicity was noted only for Nep, wherein an 87% reduction in viability was produced at a 100 μM concentration. c^{3}Nep and c^{3}Ari at 100 μM reduced viability by 34 and 21%, respectively. Intracellular levels of *S*-adenosylhomocysteine (AdoHcy) were elevated by a 24-hr exposure to 100 μM c^{3}Nep, Nep and c^{3}Ari and were 120, 75 and 25 pmoles/10^6 cells respectively. Only Nep was metabolized to an *S*-adenosylmethionine-like metabolite, and its formation was dose-related to its cytotoxicity. The $t_{1/2}$ for the disappearance of elevated levels of AdoHcy following drug removal was 1.6 to 2.5 hr for all drugs. rRNA and tRNA methylation was inhibited significantly by Nep, but c^{3}Nep and c^{3}Ari inhibited tRNA methylation but not rRNA methylation to a lesser degree. These results demonstrate that c^{3}Nep is a potent inhibitor of AdoHcy synthesis with a low degree of cytotoxicity.

The isolation and characterization of the unique cyclopentenyl analog of adenosine, Nep§, was first reported by Yaginuma *et al.* [1]. Subsequently, the novel synthesis of a series of cyclopentenyl analogs was reported by Arita *et al.* [2] and by our laboratory [3–6]. Each analog appears to be diverse in its pharmacological activity as exemplified by the ability of cyclopentenyl cytidine to inhibit CTP synthesis [7, 8] and by the inhibition of RNA methylation by Nep [9, 10]. These drugs possess antiproliferative activity against human colon carcinoma cells [7, 9], and the cyclopentenyl cytidine analog is also highly active as a differentiating agent in human promyelocytic leukemia HL-60 [8]. In further investigations of analogs in this series, we wished to develop a potent inhibitor of AdoHcy hydrolase as a potential antitumor or antiviral agent. Previous studies have determined that the carbocyclic analog of 3-deazaadenosine, c^{3}Ari, was not only a more potent and effect inhibitor of AdoHcy hydrolase [11, 12], but that it did not contain the inherent cytotoxic and less selective biological activity of the parent drug [11, 13–15]. Thus, c^{3}Nep was synthesized to obtain a potentially more active inhibitor of AdoHcy hydrolase than c^{3}Ari. In the present study, we wish to report the activity of c^{3}Nep as a cytotoxic agent against the colon carcinoma cell line HT-29 and as an inhibitor of AdoHcy hydrolase and RNA methylation in these cells.

MATERIALS AND METHODS

Materials. [^{35}S]Methionine (1115 Ci/mmole), [^{3}H-*methyl*]methionine (80 Ci/mmole) and [U-^{14}C]uridine (506 mCi/mmole) were purchased from New England Nuclear. c^{3}Ari was provided by Dr. Peter K. Chiang, Walter Reed Army Institute of Research. Nep was obtained from the Natural Products Branch, National Cancer Institute.

Synthesis of c^{3}Nep. The required 6-chloro-3-deazapurine was prepared according to the literature [16] and used as the sodium salt for the direct displacement reaction with the cyclopentenyl tosylate **1** as shown in Fig. 1. This reaction was performed under the same experimental conditions reported previously for the synthesis of Nep [6]. The combined yield of the two possible N-9 and N-7 isomers was 59%. The desired and less polar N-9 isomer (**2**) was obtained in 33% yield after column chromatography (silica gel; ethyl acetate–hexane, 1:1). The benzyl and the isopropylidene groups were simultaneously removed with BCl_3 by analogy with Nep [4, 6, 17]. At this stage, the structural assignment of the deblocked N-9 isomer (**3a**) was based on ^{1}H Nuclear Overhauser Effect (NOE) measuremonts. Through space interactions between purine protons and cyclopentenyl protons were examined by irradiation (0.032 W) of the aglycon protons and integration of the signals corresponding to the carbocyclic protons. An enhancement of 1.03 was considered to be experimentally significant. Irradiation of H-8 produced an

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§ Abbreviations: Nep, neplanocin A; c^{3}Nep, 3-deazaneplanocin A; c^{3}Ari, 3-deazaaristeromycin; AdoHcy, *S*-adenosylhomocysteine; NepMet, *S*-neplanocylmethionine; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

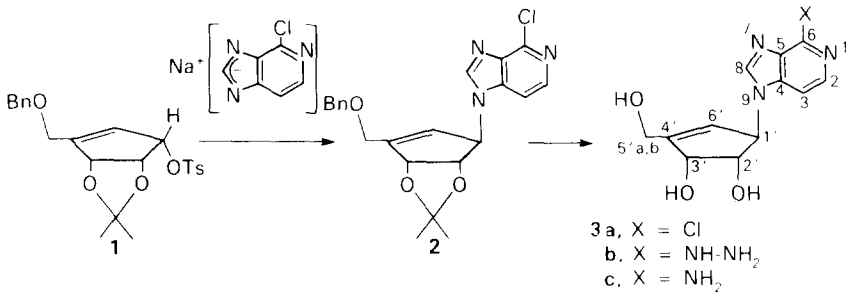

Fig. 1. Synthesis of c³Nep (**3c**).

enhancement of 1.11 of the anomeric (H-1′) signal. Irradiation of H-2 enhanced only H-3 (1.10), whereas irradiation of H-3 enhanced H-2 (1.20), H-1′ (1.10), and H-2′ (1.03). These results are in agreement with the structure for the N-9 isomer. As anticipated for the N-7 isomer, neither irradiation of H-2 nor H-3 produced any enhancement of the carbocyclic proton signals. Compound **3a** was subsequently converted to c³Nep (**3c**) by treatment with anhydrous hydrazine followed by reduction with Raney nickel. After purification by preparative C-18 reverse phase column chromatography, employing 20% aqueous methanol as eluant, the product-containing fractions were lyophilized to give c³Nep as a white powder. The u.v. spectrum of c³Nep (λ_{max} 262, pH 7) was, as expected, superimposable on that of c³Ari. The other spectral characteristics of c³Nep were in agreement with the structure: NMR (D_2O) δ 8.31 (s, 1H, H-8), 7.65 (d, J = 7.4 Hz, 1H, H-2), 7.21 (d, J = 7.4 Hz, 1H, H-3), 6.09 (bs, 1H, H-6′), 5.53 (m, 1H, H-1′), 4.69 (d, J = 5.6 Hz, 1H, H-3′), 4.41 (s, 2H, H-5′a,b), and 4.35 (m, 1H, H-2′); MS (FAB, positive mode), *m/z* (rel. intensity) 263 (MH^+, 36.7), 135 (b + 2H, 21.6); high resolution FAB MS, *m/z* 263.112 (MH^+, calcd. 263.114); $[\alpha]_D^{24}$ − 13.5° (c 0.112, H_2O).

Cell culture. HT-29 cells were maintained under an air atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 40 mM Hepes (pH 7.4), 10% heat-inactivated fetal calf serum and gentamicin, 50 μg/ml. Cell inocula consisted of 10^5 cells/10 ml of medium in 25-cm² plastic flasks (Falcon) or 3×10^5 cells/30 ml of medium in 75-cm² flasks.

Cell viability. Cells (500 or 1000) were plated into 96 well microtiter plates in 0.1 ml of medium and treated for 24 hr with the various drugs. Following this exposure interval, drug was removed, fresh medium was added, and cells were incubated further for 3 days. Viable attached cells were measured by fixation in 10% formalin, staining with 0.05% crystal violet, washing with cold phosphate-buffered saline, extracting the stained cells with 150 μl of 50% ethanol:0.1% acetic acid, and reading the absorbance at 570 nm.

AdoHcy analysis. Cells were grown in 25-cm² flasks and were labeled for 24 hr with 2 μCi/ml of [^{35}S]methionine (20 mCi/mmole) concurrently with drug treatment. Cells were harvested, washed once with ice-cold phosphate-buffered saline, and extracted in Eppendorf tubes with 0.1 ml of 5% trichloroacetic acid. Trichloroacetic acid was removed by extraction with 0.2 ml of 0.5 M trioctylamine in trifluorotrichloroethane, and the aqueous phase was injected into a Brownlee reverse-phase column and eluted with a step gradient of methanol and 0.02 M KH_2PO_4 (pH 3.8) [9]. Fractions were collected every minute for 13 min, and radioactivity was determined by scintillation counting. AdoHcy eluted at 10 min and the methionine metabolite of neplanocin (NepMet) eluted at 3 min.

rRNA and tRNA Synthesis. Cells were grown in 75 cm² flasks and labeled with 30 μCi of [^{3}H]-methionine (33 mCi/mmole) and 0.075 μCi of [^{14}C]uridine (506 mCi/mmole) simultaneously with drug treatment. After drug treatment, cells were washed with cold phosphate-buffered saline and extracted as described previously [9]. rRNA and tRNA were separated by electrophoresis in composite gels of 1.9% acrylamide and 0.6% agarose [9].

RESULTS

The cytotoxicity of c³Nep, Nep or c³Ari on human colon carcinoma cell line HT-29 was assessed following drug treatment for 24 hr using a microtiter plate assay. Nep produced a 69 and 87% reduction in viability at 10 and 100 μM concentrations respectively. In contrast, c³Nep at identical concentrations decreased cell viability by 16 and 34%, respectively, whereas c³Ari produced a 1 and 21% reduction in viability (Fig. 2).

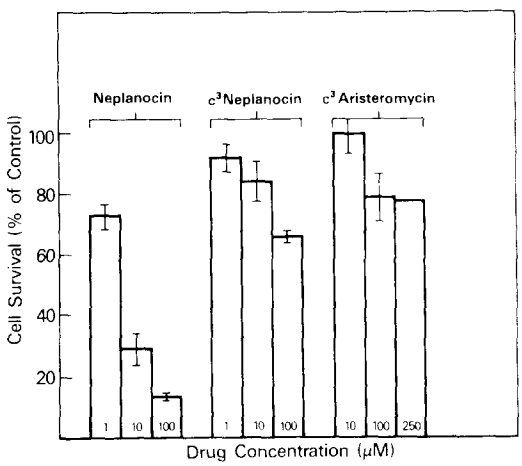

Fig. 2. Cytotoxicity of c³Nep, Nep and c³Ari against HT-29 cells after 24-hr treatment. Each value is the mean ± SE of four experiments.

Experiments were next performed to determine if c^3Nep possessed inhibitory activity against AdoHcy hydrolase *in vivo*, as previously reported for Nep [18, 19] and c^3Ari [11, 12]. Inhibitory activity against AdoHcy hydrolase in the intact cell was measured by the elevation in AdoHcy concentration in cell extracts (Fig. 3). Nep produced an increase in AdoHcy beginning at 0.1 μM drug and elevated AdoHcy concentrations to 80 pmoles/10^6 cells at 1–100 μM drug. Control levels of AdoHcy averaged 0.9 ± 0.2 pmoles/10^6 cells (mean ± SE of eleven experiments). The elevation of AdoHcy by Nep did not correlate with the cytotoxicity produced by this drug, but the level of the methionine metabolite of Nep, NepMet [9, 20], did increase in proportion to cytotoxicity and reached a concentration of 1400 pmoles/10^6 cells. c^3Nep and c^3Ari did not form a similar anabolite. c^3Nep at 100 μM elevated AdoHcy levels to 120 pmoles/10^6 cells and was 6-fold more potent at this concentration than c^3Ari.

The kinetics of AdoHcy elevation in HT-29 cells after 24-hr treatment with 1 μM Nep or 10 μM c^3Nep or c^3Ari indicated that AdoHcy was maximally elevated within 4 hr by c^3Nep, whereas maximum levels of AdoHcy were attained after 24-hr treatment with Nep or c^3Ari (Fig. 4). The $t_{1/2}$ for disappearance of AdoHcy after drug removal was similar for these carbocyclic analogs and ranged from 1.6 to 2.5 hr. The formation of NepMet in cells incubated with 1 μM Nep continually increased even after 2 hr fol-

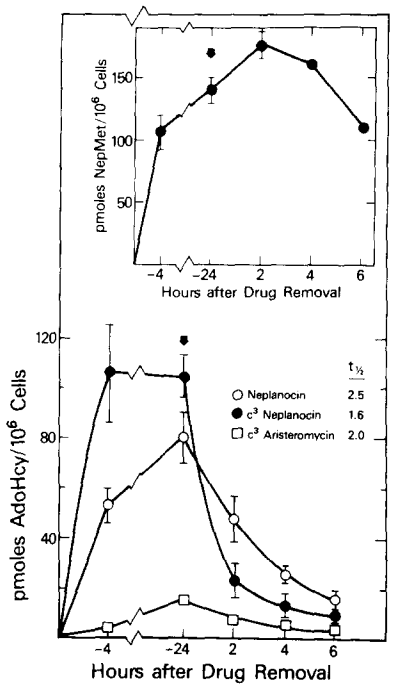

Fig. 4. Kinetics of AdoHcy formation and its disappearance following drug removal. Arrow indicates time of drug removal. Inset indicates the formation of NepMet. Each value is the mean ± SE of three experiments.

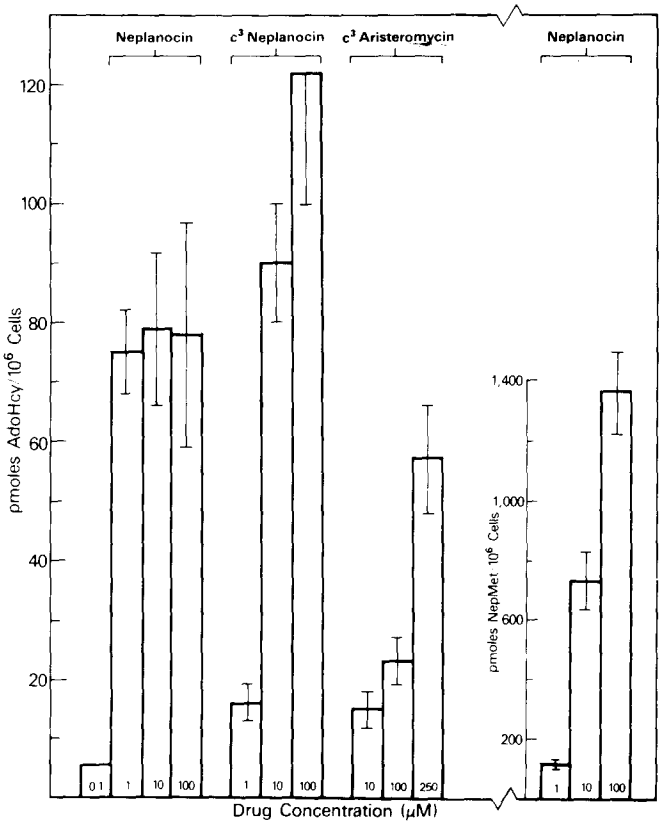

Fig. 3. AdoHcy levels in HT-29 cells treated for 24 hr with Nep, c^3Nep or c^3Ari. Also shown (right) is the level of NepMet produced by Nep treatment. Each value is the mean ± SE of six to eight experiments.

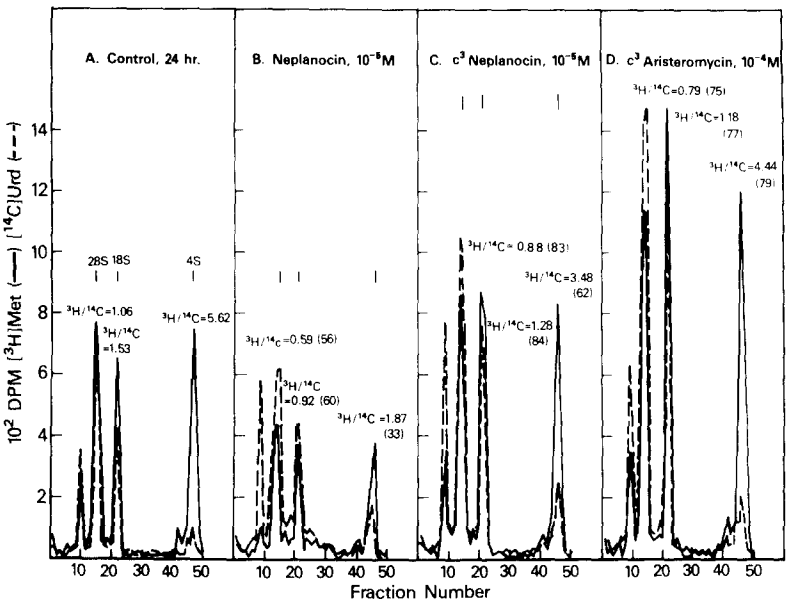

Fig. 5. Methylation and synthesis of rRNA and tRNA after 24-hr treatment with c^3Nep, Nep or c^3Ari. The ratio of $^3H/^{14}C$ represents the ratio of incorporated [^{3}H]methionine/[^{14}C]uridine in 28S, 18S and 4S RNA.

lowing drug removal and decayed more slowly than did AdoHcy.

To ascertain if elevations in AdoHcy produced a decrease in the methylation of RNA, cells were treated for 24 hr and labeled concurrently with [^{3}H]methionine and [^{14}C]uridine. Separation of rRNA and tRNA by gel electrophoresis (Fig. 5 and Table 1) indicated that tRNA methylation was more sensitive than rRNA methylation to all three drugs; however, among these analogs only Nep produced a major reduction in methylation.

Nucleoside triphosphate levels in cells treated with either 10^{-5} M Nep, 10^{-5} c^3Nep or 10^{-4} M c^3Ari were changed markedly (Table 2). However, the specific radioactivities of CTP and UTP were elevated by Nep but only marginally affected by c^3Nep and c^3Ari. The effect of Nep on CTP and UTP probably reflects the marked cytostasis produced by this drug which, in turn, results in a slower turnover of these nucleotides.

DISCUSSION

The present study has examined the pharmacological activity of a new synthetic cyclopentenyl analog, c^3Nep. In HT-29 cells, c^3Nep was nontoxic after a 24-hr exposure interval to concentrations as high as 100 μM, and only slightly inhibitory to RNA methylation. Similar results were obtained for c^3Ari, although it proved to be a weaker agent as judged by its ability to elevate AdoHcy levels. In contrast, Nep which lacks the 3-deaza modification in the

Table 1. Methylation and synthesis of rRNA and tRNA after 24 hr of treatment with Nep, c^3Nep or c^3Ari*

Treatment	$^3H/^{14}C$ ratio		
	28S rRNA	18S rRNA	tRNA
Control	1.12 ± 0.18 (100)	1.18 ± 0.23 (100)	6.60 ± 1.21 (100)
Nep, 10^{-5} M	0.69 ± 0.05 (62)	1.15 ± 0.13 (64)	2.48 ± 0.26 (38)
C^3Nep, 10^{-5} M	0.98 ± 0.13 (88)	1.56 ± 0.15 (86)	4.43 ± 0.53 (67)
c^3Ari, 10^{-4} M	0.90 ± 0.11 (80)	1.39 ± 0.22 (77)	4.09 ± 0.72 (62)

* Cells were labeled with [^{3}H]methionine and [^{14}C]uridine concurrently with drug treatment as described under Materials and Methods. The ratio of $^3H/^{14}C$ represents the ratio of incorporated [^{3}H]methionine/[^{14}C]uridine per A_{260} unit of RNA. Each value is the mean ± SE of four experiments. Numbers in parentheses indicate the percentages of control values.

Table 2. Nucleoside triphosphate levels and the specific radioactivity of CTP and UTP following treatment with c^3Nep, Nep and c^3Ari*

Treatment	Levels (pmoles/10^6 cells)				Specific radioactivity (dpm/mmole)		
	CTP	UTP	ATP	GTP	CTP	UTP	CTP + UTP
Control	660	1500	3720	1030	50	34	38
	(100)	(100)	(100)	(100)	(100)	(100)	(100)
c^3Nep, 10^{-5} M	660	1480	3540	1110	67	44	51
	(100)	(99)	(95)	(108)	(134)	(129)	(134)
Nep, 10^{-5} M	920	1670	4330	1540	186	142	157
	(139)	(111)	(116)	(150)	(372)	(418)	(413)
c^3Ari, 10^{-4} M	590	1130	3090	980	56	39	47
	(89)	(75)	(81)	(95)	(112)	(115)	(116)

* Cells were treated for 24 hr with c^3Nep, Nep or c^3Ari simultaneously with 0.075 μCi of [^{14}C]uridine, and trichlorocetic acid-soluble extracts were prepared, neutralized and chromatographed by anion-exchange HPLC as described previously [9]. Numbers in parentheses represent percentages of control values. Each value is the mean of two determinations which did not differ by more than 10%.

adenine moiety was considerably more cytotoxic and potent as an inhibitor of RNA methylation despite the fact that it produced nearly an equivalent accumulation of AdoHcy. This effect is probably a result of the conversion of Nep to the *S*-adenosylmethionine-like metabolite, NepMet, which was not evident with the other two drugs. NepMet is probably responsible, in part, for the cytotoxicity and inhibitory properties against RNA methylation produced by this drug [9]. However, it should also be noted that the triphosphate of Nep, the immediate precursor of NepMet, also accumulates in HT-29 cells [9], and thus some of the pharmacological activities of Nep may be due to as yet unidentified effects by this metabolite.

Cells treated with c^3Nep accumulated AdoHcy more rapidly than did cells treated with c^3Ari or Nep, possibly indicating a pharmacokinetic advantage of this analog. In contrast, the $t_{1/2}$ values for disappearance of accumulated AdoHcy following drug removal were similar for all three analogs. Thus, 4 hr after drug addition, c^3Nep produced a 25-fold greater accumulation of AdoHcy than did c^3Ari, whereas this difference was 7-fold after 24 hr of drug exposure and only 2-fold at 4 hr after drug removal. Previous results in HL-60 leukemia cells indicate a slightly greater accumulation of AdoHcy after Nep treatment in comparison to cells treated with c^3Nep, although both agents are considerably more effective than c^3Ari in their abilities to elevate AdoHcy levels [21].

Since elevation of host cellular AdoHcy concentrations appears to correlate with the antiviral activities of 3-deazaadenosine, c^3Ari and Nep [18, 19, 22], c^3Nep should also prove useful as an antiviral agent. Preliminary experiments indeed indicate that this new carbocylic analog possesses antiviral activity against vaccinia, polio and coxsackie viruses.

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