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N-(2-Chloroethyl)-*N*-nitrosourea Tethered to Lexitropsin Induces Minor Groove Lesions at the *p53* cDNA That Are More Cytotoxic than Mutagenic[1]

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ABSTRACT

Many different *N*-chloroethyl-*N*-nitrosourea (CENU) derivatives have been synthesized in an attempt to minimize carcinogenic activity while favoring antineoplastic activity. CENU derivatives linked to the dipeptide lexitropsin (lex) showed significant changes in groove- and sequence-selective DNA alkylation inducing thermolabile N^3-alkyladenines (N3-Alkyl-As) at lex equilibrium binding sites. CENU-lex sequence specificity for DNA alkylation was determined using ^{32}P-end-labeled restriction fragments of the *p53* cDNA. The adducted sites were converted into single-strand breaks by sequential heating at neutral pH and exposure to piperidine. To establish the mutagenic and lethal properties of CENU-lex-specific lesions, a yeast expression vector harboring a human wild-type *p53* cDNA was treated *in vitro* with CENU-lex and transfected into a yeast strain containing the *ADE2* gene regulated by a p53-responsive promoter. p53 mutants were isolated from independent *ade*$^-$ transformants. The results revealed that: (*a*) CENU-lex preferentially induces N3-Alkyl-A at specific lex equilibrium binding sites, the formations of which are strongly inhibited by distamycin; (*b*) reactivity toward Gs is still present, albeit to a lesser extent when compared to *N*-(2-chloroethyl)-*N*-cyclohexyl-*N*-nitrosourea and to CENU; (*c*) 91% of the 49 CENU-lex *p53* mutations (45 of 49) were bp substitutions, 29 of which were GC→AT transitions, mainly at 5′ purine G sites; (*d*) all AT-targeted mutations but one were AT→TA transversions; (*e*) the distribution of the CENU-lex mutations along the *p53* cDNA was not random, with position 273 (codon 91), where only GC→AT transitions were observed, being a real ($n = 3$, $P < 0.0002$) CENU-lex mutation hot spot; and (*f*) a shift in DNA alkylation sites between lesion spectra induced by CENU-lex and *N*-(2-chloroethyl-*N*-cyclohexyl-*N*-nitrosourea was associated with an increased lethality and a decreased mutagenicity, whereas no dramatic change in mutational specificity was observed. Hence, it is tempting to conclude that, in this experimental system, N3-Alkyl-A is more lethal than mutagenic, whereas O^6-alkylguanine is a common premutational lesion formed at non-lex binding sites. These results suggest that CENU derivatives with virtually absolute specificity for A residues would make targeting of lethal, nonmutagenic lesions at A+T-rich regions possible, and this may represent a new strategy for the development of new chemotherapeutic agents with a higher therapeutic index.

INTRODUCTION

CENUs[3] are important chemotherapeutic agents used for the treatment of different types of cancer (1). Unfortunately, CENUs have not only antineoplastic but also mutagenic (2–4) and carcinogenic activity and were recently classified as "probably carcinogenic to humans" (5). The antineoplastic action of CENUs is correlated to their DNA-DNA cross-linking activity. However, this type of lesions generally accounts for just a small percentage of overall alkylation. The predominant position for DNA alkylation is at N^7-guanine (~95% of total base adducts). The N^7-(2-chloroethyl)-guanine adduct may then react with the same position of a neighboring guanine, leading to a N^7-G::N^7-G intrastrand cross-link. The O^6-(2-chloroethyl)-guanine adduct (2–3% of total adducts) is considered the precursor of interstrand cross-links (≤1% of overall adduction): the O^6-monoadduct can rearrange to generate a N^1-O^6-G exocyclic intermediate, which, in turn, can undergo nucleophilic attack by the N^3-C of the cDNA strand. Quantitatively minor lesions occur on adenine and on the pyrimidine bases either as chloroethyl (or hydroxyethyl) adducts or as exocyclic rings with ethane bridges (6). Surprisingly, the generation of minor groove alkylation at N^3-adenine has not been observed for CENUs (7), whereas different monofunctional nitrosoureas and the 1-(2-hydroxyethyl)-nitrosourea react with N^3-adenine to a significant extent (8, 9).

Many different CENUs have been synthesized in the attempt to minimize carcinogenic activity while favoring antineoplastic activity. As a strategy to modify and force the DNA lesion spectrum, some CENU derivatives were linked to a series of information-reading peptides, also called lexs, which are related to the antiviral, antitumor antibiotics netropsin and distamycin (10). These drugs are able to bind the DNA minor groove by establishing close van der Waals contacts, specifically interacting with A+T-rich sequences. It has been demonstrated that CENU-lex compounds obtained through the connection of the CENU moiety to lex by a C_2H_4 linker (11) can produce sequence specific adducts targeted at nucleophilic sites not affected by simple CENUs. In particular, CENU-lex (Fig. 1) has been shown to produce new minor groove lesions, such as N^3-(2-chloroethyl)-adenine and N^3-(2-hydroxyethyl)-adenine, at lex binding sites, with a concomitant significant reduction of the alkylation at G residues in the major groove (12).

The analysis of many CENU-induced mutation spectra obtained in *Escherichia coli*, *Saccharomyces cerevisiae*, CV1 monkey cells, and CHO cells by different experimental approaches (13–21) revealed that these drugs induce mainly GC→AT transitions at Gs preceded 5′ by a purine, suggesting that O6-Alkyl-G is the main responsible for such bp substitutions. The 5′-flanking purine effect has been explained as the consequence of a structural influence on the initial formation of O6-Alkyl-G, which may be further enhanced by sequence-dependent variation in alkylation-specific repair (22). In contrast, with melphalan (L-phenylalanine mustard), the predominant bp substitutions induced were AT→TA transversions (23). The same authors showed that all AT bp that were frequent sites for melphalan-induced AT-targeted transversions were also prominent sites of thermolabile adenine adducts (N3-Alkyl-A), although some sites for adduction were cold spots for mutations (24).

Because no data are available on the mutagenic properties of CENU-lex-specific lesions, *i.e.*, adenine-targeted lesions at lex binding sites, we determined at the nucleotide level the distribution of thermolabile adenine adducts induced by CENU-lex and the mutation

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[3] The abbreviations used are: CENU, *N*-chloroethyl-*N*-nitrosourea; lex, lexitropsin; O6-Alkyl-G, O^6-alkylguanine; N3-Alkyl-A, N^3-alkyladenine; CCNU, *N*-(2-chloroethyl-*N*-cyclohexyl-*N*-nitrosourea; 3-Methyl-A, N^3-methyladenine.

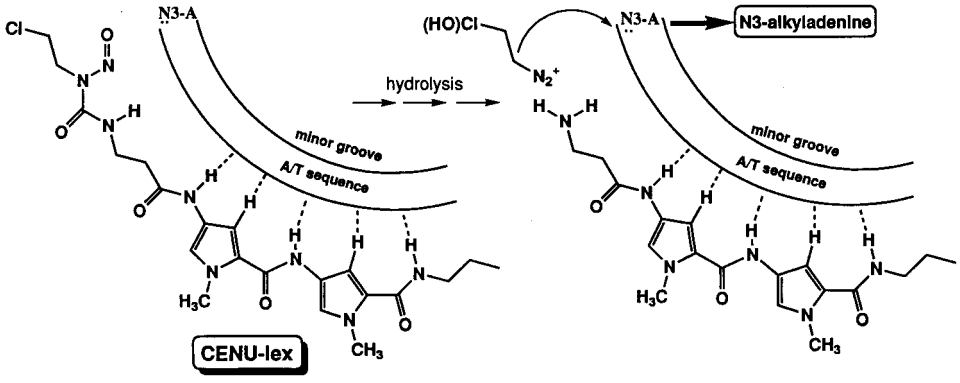

Fig. 1. Equilibrium binding of CENU-lex to DNA and formation of N3-Alkyl-A.

spectrum of this drug. The human *p53* tumor suppressor gene cDNA inserted in a yeast expression vector was used as a target. The sequence specificities for DNA alkylation by CENU-lex, CCNU, and CENU were determined through the conversion of the adducted sites into single-strand breaks by sequential heating at neutral pH and exposure to base using ^{32}P-end-labeled restriction fragments of the *p53* cDNA. The lethal and mutagenic effects of CENU-lex have been evaluated by using an *in vitro* mutagenesis protocol (21) and exploiting a repair-proficient haploid *S. cerevisiae* strain for the processing of DNA lesions into mutations (25).

MATERIALS AND METHODS

Compounds. Unless stated otherwise, reagents of the highest purity were purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich (Milwaukee, WI). CENU-lex was prepared as described previously (11). Restriction enzymes, phosphatases, kinases, and DNA polymerases were obtained from New England Biolabs (Beverly, MA).

Preparations of p53-radiolabeled Restriction Fragments. Plasmid pLS76 containing the p53 cDNA, provided by Dr. R. Iggo (ISREC, Epalinges, Switzerland) was amplified in One Shot Tm INV αF′ cells (Invitrogen, San Diego, CA), purified and restricted to obtain fragments including positions 169–667 (*Psh*AI site, position 169; *Nco*I site, position 476; *Bsu*36I site, position 667). The *Psh*AI/*Nco*I DNA fragment [5′-^{32}P-labeled on transcribed (noncoding) strand at the *Nco*I site] was obtained using *Nco*I restriction endonuclease, phosphatase, and kinase treatment, followed by a final *Psh*AI digestion and polyacrylamide gel purification. The 5′-^{32}P-end-labeled *Nco*I/*Bsu*36I DNA fragment [5′-^{32}P-labeled on nontranscribed (coding) strand at the *Nco*I site] was obtained by initial endonuclease restriction with *Nco*I, followed by sequential treatment with calf intestine alkaline phosphatase, phosphorylation with T4-kinase in the presence of [γ-^{32}P]ATP. The DNA was then restricted with *Bsu*36I and the labeled fragment isolated from a 6% polyacrylamide gel. The same procedure was used to prepare the 5′-^{32}P-end-labeled *Bsu*36I/*Nco*I DNA fragment [5′-^{32}P-labeled on transcribed (noncoding) strand at the *Bsu*36I site], except that the order of enzyme digestion was reversed.

Alkylation Reactions. The p53 5′-labeled restriction fragments (200,000 cpm) and sonicated calf thymus DNA (83 μM final concentration) in 10 mM Tris-1 mM EDTA buffer (pH 7.6) were incubated with CENU-lex, CCNU, or CENU (final concentrations in figure legends) in the presence or absence of 100 μM distamycin for 2 h at 37°C. DNA was ethanol-precipitated and washed with cold 70% ethanol. The DNA pellet was dissolved in 30 μl of 10 mM Tris buffer (pH 7.0) and heated at 90°C for 15 min. The DNA was then reprecipitated, washed as described above, and dried under vacuum. The pellet was resuspended in 100 μl of 1 M piperidine and heated at 90°C for 30 min. The piperidine was removed by repeated lyophilization, and the DNA was resuspended in 10 μl of 80% formamide (v/v), 50 mM Tris-borate buffer (pH 8.3), 1 mM EDTA, 0.1% xylene cyanol (w/v), and 0.1% bromphenol blue (w/v). The radioactivity in an aliquot from each sample was determined; the remaining sample was denatured (3 min at 90°C) and then chilled in ice water. An equivalent amount of radioactivity for each reaction was loaded on a 12% polyacrylamide denaturing gel (7.78 M urea) and run at 65 W constant power. For sequence markers, lanes containing Maxam-Gilbert G and G + A (and, in some cases, C and C + T) reactions were included in each gel. The gels were dried and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Vectors, Strain, and Media. The yeast expression vector pLS76, harboring a human wild-type *p53* cDNA under the control of an ADH1 promoter and containing the LEU2 selectable marker (26), was used in the *in vitro* damaging treatment. Plasmid pRDI-22 was used for GAP repair assays (25). pRDI-22 is identical to pLS76, except for the presence of a linker cloned between the p53 *Bsm*I and *Stu*I sites. pRDI-22 cut with *Hin*dIII and *Stu*I can be used for GAP repair assays without gel purification. The haploid repair-proficient *S. cerevisiae* strain *yIG397* (*MAT***a** *ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ura3-1 URA3 3×RGC::pCYC1::ADE2*) was used as recipient of pLS76 and for GAP repair assays. The p53-dependent reporter *ADE2* gene (25) allowed the phenotypic selection of p53 mutants. Standard yeast manipulations were performed as described (27).

DNA Modification, Analysis, and Transfection. CENU-lex was dissolved in absolute ethanol immediately before the treatment. Plasmid pLS76 DNA (1.5 μg) was treated with different CENU-lex concentrations (Table 1) in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 50% ethanol for 1 h at 37°C. DNA was purified by three ethanol precipitations, washed with 70% ethanol, and resuspended in sterile water. Damaged or undamaged (mock-treated) vectors were then transfected into yIG397 cells by electroporation and transformants were plated on selective synthetic medium containing 1 M sorbitol. After 3 days of incubation at 30°C, colonies appeared. The selection for the plasmid marker (LEU2) allowed an indirect determination of the lethal effect of the damaging treatment as the number of transformants scored in transfections with damaged plasmids with respect to that obtained with undamaged vector. In other words, it is assumed that the decrease in transfection efficiency (number of transformants/μg of plasmid) observed after the damaging treatment is due to the fact that lethal lesions present on the damaged plasmid, if they are not repaired, will not allow its replication. As transformation plates contained a minimal amount of adenine, adenine auxotrophs were able to achieve only a few cell divisions and, thus, produced smaller red colonies. Spontaneous and induced mutant frequencies were defined as the number of small red colonies with respect to the total number of transformants. The fold of mutant induction was defined as the ratio between the mutant frequency

Table 1 *Survival and mutation induction in undamaged and CENU-lex-damaged pLS76 after passage through yIG397 strain*

CENU-lex (mM)	Survival (%)	*p53* mutant frequency	MFR[a]
0	100 (n = 16)[b]	3.3×10^{-4} (16/47,821)	1
0.2	43 ± 6 (n = 6)[b]	9.8×10^{-4} (6/6,087)	3
0.4	39 ± 19 (n = 6)[b]	22×10^{-4} (36/16,366)	6.7
1	11 ± 10 (n = 15)[b]	63×10^{-4} (135/21,451)	19

[a] MFR, mutant frequency of CENU-lex-damaged DNA/mutant frequency of undamaged DNA.

[b] n, no. of independent transfections, *i.e.*, no. of transfections performed with different aliquots of damaged (or undamaged) plasmid DNA.

observed with damaged vector with respect to the spontaneous frequency. Phenotypic mutant clones were purified and characterized at the molecular level as described previously (21). To determine whether adenine auxotrophy was due to *p53* mutations rather than, for example, mutations in the promoter, the *p53* open reading frame between nucleotide positions 125 and 1122 from each rescued plasmid was PCR-amplified using primers P3 and P4 and *Pfu* DNA polymerase (Eppendorf, Milano, Italy) as described previously (25). Unpurified PCR products and *Hin*dIII-*Stu*I-linearized pRDI-22 were cotransfected by electroporation into yIG397. The *Hin*dIII-*Stu*I-digested pRDI-22 gapped plasmid has two regions homologous to the terminal regions of the PCR product obtained using P3 and P4. After cotransfection and followed by homologous recombination *in vivo* (GAP repair) a p53 expression vector is reconstituted having the wild-type promoter region (derived from pRDI-22) and the core region of the p53 open reading frame (derived from the P3/P4 PCR product). If the clone was initially *ade*$^-$ due to a mutation in the promoter region, the GAP-repaired transformants will originate almost exclusively white normal size colonies on limiting adenine plates (no small red colonies) because the promoter of in the GAP-repaired plasmid derived from pRDI-22 is wild type (GAP repair negative). On the contrary, if the PCR product contains a single mutation, ~100% of the derived transformed clones will give rise to small red colonies (GAP repair positive; see Fig. 1 in Ref. 21). p53 mutants, giving ~100% red colonies (GAP repair positive), were reamplified and sequenced as described previously (21). Only independent mutants were included in the mutation spectrum; those carrying the same genetic alterations were considered independent only if they were isolated from different (independent) transfections. To compare lethality and mutagenicity of CCNU- and CENU-lex-induced lesions and to avoid a comparison based on molar concentration, which can be influenced for example by differences in solubility and reactivity, the relative mutagenicity:lethality ratio was used. For each damaging treatment a ratio between mutagenicity observed over the level of survival reached was determined.

RESULTS

***In Vitro* Alkylation Patterns.** The sequence-dependent DNA alkylation by CENU-lex, CCNU, and CENU in different p53 restriction fragments is shown in Figs. 2–4. In some cases, the bands for A lesions were overexposed to allow the visualization of the much weaker bands at Gs. The assignment of the cleavage sites was done using gels analyzed under conditions of lower exposure. The sequential neutral thermal hydrolysis followed by hot piperidine treatment liberates *N*-alkyl purines from the DNA and allows the simultaneous mapping of both N^7-alkylguanine and N3-Alkyl-A.

The gel data confirm that both CCNU and CENU alkylate only at Gs with a sequence specificity consistent with that previously determined in other restriction fragments (11, 12, 28). At equal concentrations, CENU-lex is ~50% less efficient at alkylating Gs than CCNU or CENU, although the sequence specificity for reaction at G is identical for all three nitrosoureas. For example, the alkylation pattern at the G_3 run ($G_{423-425}$; Fig. 2) shows the central G being the dominant site of modification for all three compounds. This pattern was previously observed for other alkylating agents that react via cationic intermediates (Ref. 11 and references therein).

In sharp contrast with CCNU and CENU, the lesions induced by CENU-lex predominate at As, with G cleavages being significantly weaker. For example, the intensity of the band at A_{613} with 0.5 mM CENU-lex is at least 40-fold more intense than that at G_{624} (Fig. 3). With CCNU and CENU, the G_{632} is at least 500-fold more intense than that at A_{613}, whereas with CENU-lex, the A_{613} band appears to be 25-fold more intense than that at G_{632} with the same concentration of CCNU or CENU (Fig. 3). Moreover, the cleavages at As are restricted at A/T runs at least 3 bp long: isolated A residues are not targeted by CENU-lex. In most cases, the alkylation sites are at A/T runs at least 4 bp long, but there is a very strong cleavage site at A_{553}, which has a sequence of 5′-GATA$_{553}$G (Fig. 4). Importantly, the intense bands at As are inhibited by the coaddition of distamycin,

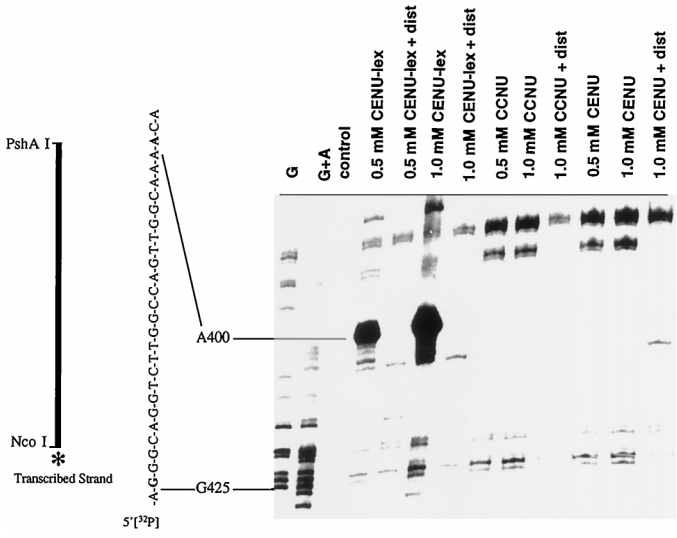

Fig. 2. Cleavage of the 5′-^{32}P-end-labeled *Psh*AI/*Nco*I DNA fragment [5′-^{32}P labeled (*) on transcribed (noncoding) strand at the *Nco*I site] of the *p53* cDNA, induced by CENU-lex, CCNU, and CENU at the indicated concentrations in the presence or in the absence of distamycin, by sequential heating at neutral pH and exposure to hot piperidine. Bases indicated in the gel are reported in *boldface* in the sequence.

which also binds in the minor groove to A+T-rich regions (10). Concomitant with the quantitative inhibition of alkylation at As by distamycin, there is an increased alkylation at Gs (Figs. 2–4). As noted previously, the alkylation at Gs by the non-equilibrium-binding CCNU and CENU is also affected by distamycin due to an electrostatic effect; however, the inhibition is much weaker and shows no sequence dependency (11).

On the basis of the alkylation pattern, CENU-lex shows an orientation preference within its equilibrium binding site. For example, at the $A_{400-403}$ sequence (Fig. 2), there is a strong preference for alkylation at the 3′-A. The same preference for the 3′-A at an A_4 run is also evident at A_{633} or at the A/T stretch at $A_{613-617}$ (Fig. 3).

Induction of p53 Mutants by CENU-lex Treatment. Plasmid pLS76 was damaged *in vitro* with increasing CENU-lex concentrations (Table 1). Damaged or undamaged (mock-treated) plasmids were transfected into *yIG397*. Transformants were selected on plates containing sufficient adenine for adenine auxotrophs to grow and turn red. The lethal effect of DNA modifications was estimated from the number of transformants appearing onto selective plates. Survival showed a CENU-lex concentration-dependent decrease, whereas the mutant frequency increased in a concentration-dependent way (Table 1). Only 1 mM-induced mutants were purified for the molecular analysis as the level of induction (19-fold above background) guaranteed that 95% of the mutants were actually induced by the drug. Only independent mutants were included in the mutation spectrum; those carrying the same genetic alterations were considered independent only if they were isolated from different (independent) transfections.

Identification of CENU-lex-induced p53 Mutations in Red Yeast Colonies. To determine whether adenine auxotrophy was due to *p53* mutations rather than, for example, mutations in the promoter, the p53 expression plasmid was rescued from 109 *ade*$^-$ *leu*$^+$ colonies and the *p53* open reading frame was PCR-amplified using the high-fidelity *Pfu* DNA polymerase and tested by GAP repair (see Fig. 1 in Ref. 21 for basic concept of GAP repair analysis). Nineteen mutant clones were negative to amplification. Ninety mutants were analyzed by GAP repair, 19% (17 of 90) of which showed a low percentage of small red colonies (GAP repair negative), indicative of the absence of p53-inactivating mutations in the DNA template.

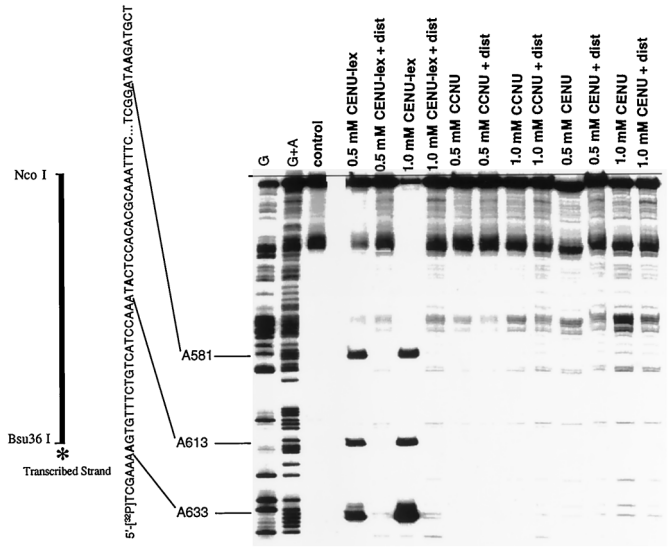

Fig. 3. Cleavage of the 5′-^{32}P-end-labeled *Nco*I/*Bsu*36I DNA fragment [5′-^{32}P-labeled (*) on the nontranscribed (coding) strand at the *Nco*I site] induced by CENU-lex, CCNU, and CENU at the indicated concentrations in the presence or in the absence of distamycin, by sequential heating at neutral pH and exposure to hot piperidine. Bases indicated in the gel are reported in *boldface* in the sequence.

Forty-seven p53 mutants showing ~100% small red colonies in the GAP repair assay (indicative of the presence of a mutation in the p53 open reading frame) were sequenced. At least one molecular alteration was found in each of them (Table 2). The large majority of mutants contained a single bp substitution (41 of 47; 87%). Forty-one different nucleotides were affected by mutation, three of which have not been found mutated thus far in tumors or cell lines (9378 mutations, IARC data base; Ref. 29).

The effective target size where an incoming mutation is phenotypically selectable along the *p53* cDNA is large: 542 bp according to an estimate with a yeast-based functional assay (30) and at least 450 considering the mutations found in tumors and cell lines (29). Hence, finding three or even two identical independent mutations affecting the same nucleotide is a rare event on the basis of random distribution ($n = 2$, $P < 0.006$; $n = 3$; $P < 0.0002$, Poisson's normal distribution). To be conservative, at least position 273 (5′-TG*g*-3′, codon 91), with three independent mutant clones, should be considered a real CENU-lex induced mutation hot spot (Table 2).

Forty-seven mutants evidenced 49 independent mutations (Table 3). Clone 22 and clone 100 each showed two bp substitutions, whose distance on the cDNA suggested an independent origin. Except for four frameshifts, all mutations were bp substitutions, 38 of which (84%) were GC-targeted. GC→AT transitions represented the predominant class of bp substitutions (29 of 45; 64%). Seventy % of GC→AT transitions involved a guanine preceded 5′ by a purine (Table 4). Six of the seven AT-targeted mutations observed were AT→TA transversions.

The 47 mutant plasmids encoded 42 different p53 mutations at the protein level, 5 of which have not been reported thus far in human tumors or cell lines (29). Thirteen mutants (13/47; 28%) showed a premature stop codon.

DISCUSSION

CENU-lex is a CENU derivative with an alkylnitrosourea moiety linked to the dipeptide lex. This drug has been designed to preferentially target at N^3 of adenine in DNA and generate lesions that are expected to be cytotoxic rather than mutagenic. In this work, we determined both the localization of lesions and the mutation spectrum induced by CENU-lex at the human *p53* cDNA. The data allow us to determine whether the adduct frequency is predictive of the mutation spectrum. Furthermore, by comparing the results obtained with CCNU and CENU-lex using the same experimental approach, we obtained insights into the mutagenic and/or toxic properties of CENU-lex-specific lesions, *i.e.*, adenine-targeted lesions at lex binding sites.

CENU-lex preferentially damages 3′-adenine in runs of three or four A or (A/T) residues. Reactivity toward Gs is still present when compared to other CENUs, although to a lesser extent (Figs. 2–4). The lesions at A's are due to the equilibrium binding properties of lex: these lesions are, indeed, absent in DNA treated with CENUs lacking the lex moiety (CCNU and CENU) and their formation by CENU-lex is strongly inhibited by distamycin, a binding site specific competitor. Reactivity at these sites is consistent with the mode of lex-DNA interaction determined by a series of structural studies (cited in Ref. 31). Although such information on lex binding to DNA provides a basis to rationalize experimentally verified binding domains, it is not possible to predict *a priori* which A+T-rich regions will be preferred binding sites within large DNA fragments. Therefore, it is not surprising that not all of the A or $(A/T)_n$ runs, were damaged by CENU-lex. This fact suggests that other factors influence the binding equilibrium between CENU-lex and DNA and could make CENU-lex a chemical probe for the fine structure of A+T-rich regions.

The localization of adenine lesions and mutations at the *p53* cDNA sequence is displayed in Fig. 5. Although many As were abundantly damaged by CENU-lex, only a few A-targeted mutations were recovered at those sites. Why are such hot spots for lesion formation so cold for mutagenesis? A plausible interpretation is that adenine lesions are not potent premutagenic lesions and/or they are more lethal than mutagenic. In addition, it is possible that mutations induced at particular sites will not cause any phenotypic change. Although representing a quantitatively minor lesion, 3-Methyl-A adducts have been shown to be able to block *E. coli* and yeast DNA replication, and, if the damage is not repaired, to kill these microbial cells (32, 33). A 3-Methyl-A-DNA-glycosylase activity from *S. cerevisiae* was identified as the product of the *MAG* gene (33). *MAG* gene product has been

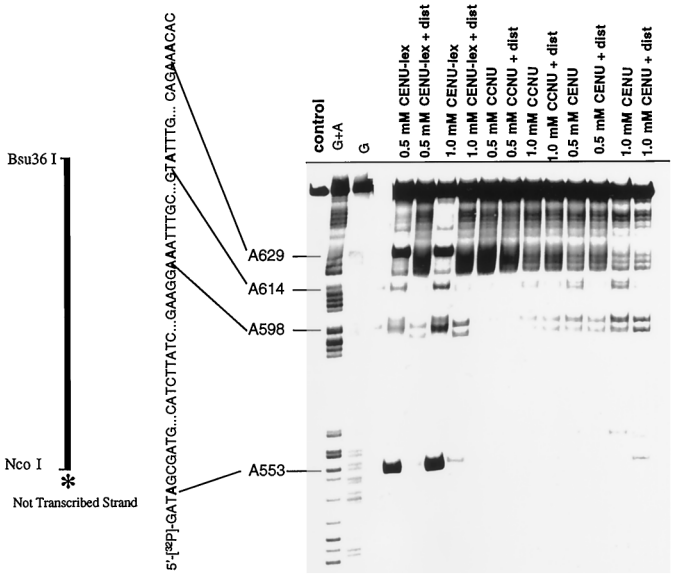

Fig. 4. Cleavage of the 5′-^{32}P-end-labeled *Bsu*36I/*Nco*I DNA fragment [5′-^{32}P-labeled (*) on the transcribed (noncoding) strand at the *Bsu*36I site] of the *p53* cDNA, induced by CENU-lex, CCNU, and CENU at the indicated concentrations in the presence or in the absence of distamycin, by sequential heating at neutral pH and exposure to hot piperidine. Bases indicated in the gel are reported in *boldface* in the sequence.

Table 2 *CENU-lex-induced mutations at the p53 cDNA after in vitro treatment of plasmid pLS76 and passage into yIG397 cells*

Mutant no.[a]	Mutation type[b]	Nucleotide position	5′→3′ sequence[c] (NT strand)	Amino acid change	CCNU[d]
Single bp substitutions					
9	GC→AT	263[e]	AGcCCC→AGTCC	A 88 V[f]	−
8, 116, 117	GC→AT	273	TGgCC→TGaCC	W 91 Stop	−
77	GC→TA	293[e]	CCcTT→CCaTT	P 98 H[f]	−
34, 108	GC→TA	296[e]	TTcCC→TTaCC	S 99 Y[f]	−
105	GC→AT	310	ACcAG→ACtAG	Q 104 Stop	−
78	AT→TA	400	TGtTT→TGaTT	F 134 I	−
39, 99	GC→AT	438	TGgGT→TGaGT	W 146 Stop	−
94	GC→AT	451	CAcCC→CAtCC	P 151 S	−
46	GC→TA	452	ACcCC→ACaCC	P 151 H	−
12	GC→TA	454	CCcCG→CCaCG	P 152 T	−
60	GC→AT	455	CCcGC→CCtGC	P 152 L	−
30	AT→TA	484	CCaTC→CCtTC	I 162 F	−
107	GC→AT	529	GCcCC→GCtCC	P 177 S	−
96	GC→AT	535	ACcAT→ACtAT	H 179 Y	−
57, 111	GC→AT	538	ATgAG→ATaAG	E 180 K	−
15	GC→AT	542	GCgCT→GCaCT	R 181 H	−
125	AT→TA	613	GtATT→GaATT	Y 205 N	−
37	AT→TA	647	TGtGG→TGaGG	V 216 E	−
95	GC→AT	730	TGgGC→TGaGC	G 244 S	+
115	GC→AT	733	GCgGC→GCaGC	G 245 S	+
14	GC→AT	734	CGgCA→CGaCA	G 245 D	+
56	AT→TA	736	GCaTG→GCtTG	M 246 L	−
50	GC→AT	749	GCcCA→GCtCA	P 250 L	−
32	GC→AT	754	TCcTC→TCtTC	L 252 F	−
98	AT→GC	770	ACtGG→ACcGG	L 257 P	−
97	GC→AT	796	TGgGA→TGaGA	G 266 R	+
27	GC→AT	797	GGgAC→GGaAC	G 266 E	−
109	GC→TA	820	GTgTT→GTtTT	V 274 F	−
70	GC→AT	827	TGcCT→TGtCT	A 276 V	+
36	GC→AT	836	TGgGA→TGaGA	G 279 E	+
92	GC→TA	836	TGgGA→TGtGA	G 279 V[f]	−
45	GC→CG	843	GAcCG→GAgCG	D 281 E	−
101	GC→AT	845	CCgGC→CCaGC	R 282 Q	−
41	GC→AT	856	AGgAA→AGaAA	E 286 K	−
124	GC→AT	916	AGcGA→AGtGA	R 306 Stop	−
25	AT→TA	981	TAtTT→TAaTT	Y 327 Stop	−
Other mutations					
42	+1 A/T	355–356	A GCC A→A GtC CA	119 frameshift	
64	−1 G/C	690	ACc ACC→ACA CC	231 frameshift	
47	−1 A/T	838	aGA GAC→GAG AC	280 frameshift	
1	+1 A/T	894–895	GAG CTG→GAG aCT	299 frameshift	
100	GC→AT	263[e]	AGcCC→AGtCC	A 88 V[f]	−
	GC→TA	796	TGgGA→TGtGA	G 266 Stop	−
22	GC→AT	381	TCcCC→TCtCC	S 127 S	−
	GC→AT	749	GCcCA→GCtCA	P 250 L	−

[a] Independent mutants showing the same mutations are identified by their number.
[b] Mutations at the GC (or AT) bp are reported as induced by G- (or A-) targeted lesions.
[c] Lowercase letters, mutated bases; underlined letters, p53 codon.
[d] +, the same amino acid substitution was found among CCNU-induced *p53* mutations (21).
[e] Base in the p53 cDNA for which bp substitutions have not been found so far in human tumors (IARC p53 database: 9378 mutations; Ref. 29).
[f] Amino acid change not found so far in human tumors or cell lines (IARC p53 data base: 9378 mutations; Ref. 29).

Table 3 *Molecular features of CENU-lex-induced p53 mutations selected in yIG397*

	No.	(%)
Total no. of mutations	49	(100)
bp substitutions	45	(92)
GC-targeted:	38	(84)
GC→AT	29	(64)
GC→TA	8	(18)
GC→CG	1	(2)
AT-targeted:	7	(16)
AT→GC	1	(2)
AT→TA	6	(13)
Frameshift	4	(8)
+1 A/T	2	(4)
−1 A/T	1	(2)
−1 G/C	1	(2)

shown to protect yeast from CENU-induced cell killing (34), to recognize 3-Methyl-A and other minor lesions such as 1-N^6-etheno-A and also to act on N^7-modified guanines (35). Given the broad substrate specificity of the yeast (33) and mouse (36), 3-Methyl-A DNA glycosylases these results do not provide conclusive evidence of N3-Alkyl-A cytotoxicity. However, in a recent study (37), *Aag* null mouse embryonic stem cells proved very sensitive to killing by a MeOSO2-C_2H_4-lex conjugate, an almost exclusive N^3-adenine alkylator (31). Furthermore, by combining a chemical and a genetic approach it has been elegantly shown that nonrepaired 3-Methyl-A induces sister chromatid exchanges, chromosome aberrations, S-phase arrest, p53 induction, and apoptosis (38).

Abundantly alkylated A-sites could appear as cold spots for mutation because a change at the DNA level will not produce any amino acid or phenotypic change. Assuming that N3-Alkyl-A (or the derived abasic site) is a lethal, noncoding lesion, the insertion of an A opposite the lesion during DNA replication ("A rule") will

Table 4 *Distribution along the p53 sequence of CENU-lex-induced GC→AT transitions: influence of DNA sequence context*

	No.	(%)
GC→AT	29	(100)
5′PuG	20	(69)
5′-GG	19	
5′-AG	1	
5′PyG	9	(31)

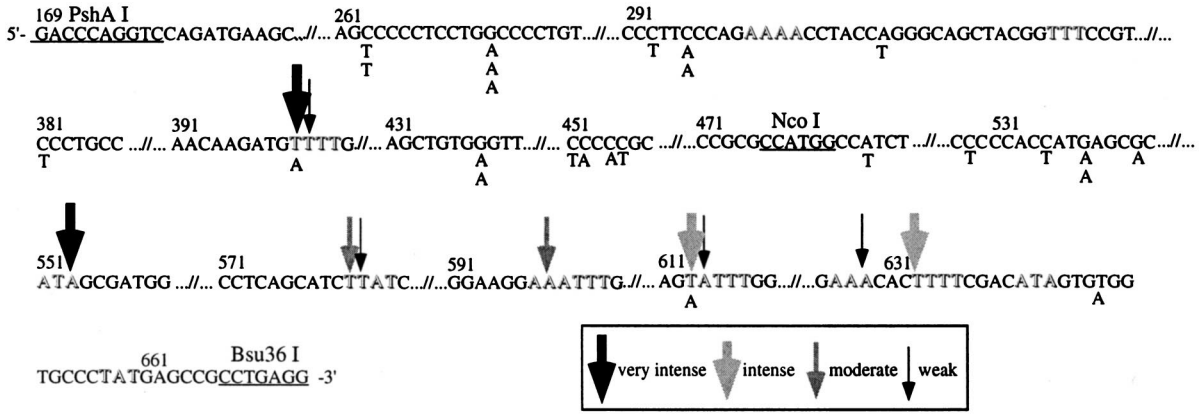

Fig. 5. Localization and band intensity of CENU-lex A-specific cleavage sites and induced mutations in the *Psh*AI/*Bsu*36I DNA fragment of the *p53* cDNA sequence [nontranscribed (coding) strand] are reported above and below the sequence, respectively. Putative lex binding sites, *i.e.*, A_n or $(A/T)_n$ ($n > 3$) are *shadowed*. For simplicity, G lesions are not reported.

eventually produce AT→TA transversions. This assumption is reasonable because more than 60% of bp substitutions induced by another alkylating agent (melphalan) were AT→TA mutations, the origin of which was attributed to N3-Alkyl-A (24). Positions 400 (Phe^{134}), 553 (Ser^{185}), 581 (Leu^{194}), 597 (Gly^{199}), 613 (Tyr^{205}), and 633 (Thr^{211}) were the most damaged A residues. AT→TA transversions were found at positions 400 and 613. For all of the other positions no mutation was observed. It can be predicted that positions 597 and 633 were cold spots for mutation because all possible bp substitutions will invariably produce synonymous codons. An AT→TA transversion at position 553 will cause a Ser^{185}→Cys substitution. The fact that this mutation has not been reported thus far among the 9378 mutations present in the p53 data base might suggest that it causes no phenotypic change. AT→TA transversions at positions 581 and 614 will cause amino acid changes (Leu^{194}→His; Tyr^{205}→Phe) already found in tumors or cell lines. However, such mutations represent less than 14 and 3% of the mutations reported at those positions, respectively. Together, these considerations suggest why CENU-lex hyperreactive As, where N3-Alkyl-A are likely formed, can be cold spots for mutations. Furthermore, sequence-dependent factors other than adduct frequency, such as efficiency of repair or of replicative bypass, probably account for the majority of the variation of mutation frequency at different sites.

The comparison between the results obtained with CCNU (Table 1 in Ref. 21) and with CENU-lex (Table 1 in this study) reveals that, at equal survival levels, the CCNU-induced mutant frequency is ~3-fold higher than that observed for CENU-lex. Conversely, at the same level of mutant frequency induction, CENU-lex is ~3-fold more toxic than CCNU. Moreover, although CENU-lex and CCNU showed a significantly different DNA reactivity (Figs. 2–4), mutational specificities of these drugs are very similar. In both cases the majority of mutations are GC→AT transitions at 5′-PuG. Hence, it is tempting to conclude that in this experimental system N3-Alkyl-A is mainly a lethal lesion, whereas O6-Alkyl-G is a common premutational lesion formed at non-lex binding sites. Consistent with this interpretation the CENU-lex mutation hot spot (position 273) is not at a lex binding site. An important corollary of this conclusion would be that CENU-lex derivatives with virtually absolute specificity for As would combine the highest cytotoxicity with the lowest mutagenicity and may represent a new strategy for the development of new chemotherapeutic agents with a higher therapeutic index. The importance of the N^3 atom of the purine ring for stabilizing the contact between DNA polymerases and its template (39–41) is likely responsible for the toxic (Ref. 38 and this study) nature of this rather subtly modified DNA base and further support our interpretation.

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