Structural Identification and Biological Activity of 7-Methyl-10,11-Ethylenedioxy-20(*S*)-Camptothecin, a Photodegradant of Lurtotecan

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ABSTRACT

An additional chromatographic peak was observed in plasma samples of patients receiving NX 211, a liposomal formulation of the topoisomerase I inhibitor lurtotecan. We have isolated and purified this product by sequential solid-phase extractions, and we report its structure and cytotoxicity relative to lurtotecan and related agents. Nuclear magnetic resonance data indicate that cleavage of the piperazino moiety occurred at the N-C bond of the B-ring, yielding 7-methyl-10,11-ethylenedioxy-20(*S*)-camptothecin (MEC). Tests of the growth inhibition potential of MEC in seven human tumor cell lines showed that the compound was approximately 2–18-fold more cytotoxic than lurtotecan, topotecan, and 7-ethyl-10-hydroxy-20(*S*)-camptothecin (SN-38). Subsequently, we found that MEC was the product of rapid photolysis of lurtotecan, with the rate of degradation inversely proportional to NX 211 concentrations, and greatly depends on light intensity. Furthermore, MEC concentrations were found to increase significantly in plasma samples exposed to laboratory light but not in blood. MEC was not produced from NX 211 in the presence of human liver microsomes, suggesting that it is not a product of cytochrome P-450 metabolism. Using a validated analytical method, trace levels of MEC were quantitated in blood samples of two patients. These observations confirm that the precautions for protection from light currently specified for preparation and administration of NX 211 dose solutions are critical. Procedures to minimize formation of MEC, by the use of amber vials for NX 211 and by preparation of dilutions immediately before clinical use in a fashion completely protected from light, are now being routinely implemented.

INTRODUCTION

The currently known analogues of camptothecin, a cytotoxic plant alkaloid from *Camptotheca acuminata*, share a basic pentacyclic structure with a chiral center located at C20 in the terminal E-ring (Fig. 1). Extensive studies on the synthesis of these derivatives and the development of structure-activity relationships have been carried out over the last several years, and some important general relationships have emerged (1). One of the principal chemical features of this class of agents is the presence of a lactone functionality in the E-ring, which is not only essential for antitumor activity but also confers a degree of instability to these agents in aqueous solutions (2). The camptothecins can undergo a pH-dependent reversible interconversion between this lactone form and a ring-opened carboxylate (or hydroxy acid) form, of which only the former exerts the characteristic topoisomerase I-inhibitory activity. In the search for more potent camptothecin analogues, the synthetic preparation of hexacyclic derivatives has been described, and two representative agents of this class, lurtotecan and exatecan mesylate (DX-8951f), are currently undergoing clinical evaluation (3, 4). The former is a water-soluble derivative with a dioxalane moiety between C10 and C11 (5) and has been evaluated clinically in various Phase I and Phase II trials using a 30-min i.v. infusion given daily for 5 days or as a 72-h continuous i.v. infusion (6–8). Alternative strategies of lurtotecan administration are currently being developed, including a new liposomal formulation, NX 211, to further stabilize the lactone moiety and induce sustained drug levels that may be required to maximize antitumor activity (9, 10). We previously reported the presence of an unidentified compound in the plasma of patients treated with NX 211 as well as in control samples with the liposomal product added (11). Because the native fluorescence intensity of the parent drug (*i.e.*, lurtotecan) was shown previously to be sensitive to UV-light exposure (254 nm) and the fact that the peak height of the newly generated compound was correlated with the lurtotecan concentration, we suspected photodegradation of lurtotecan to produce an additional species. In the current study, we have isolated this unknown compound by using HPLC[2] coupled with fluorometric detection, followed by NMR analysis of the purified fraction.

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[2] The abbreviations are: HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PCA, perchloric acid; MEC, 7-methyl-10,11-ethylenedioxy-20-(*S*)-camptothecin; SN-38, 7-ethyl-10-hydroxy-20-(*S*)-camptothecin; ACN, acetonitrile; D5W, 5% (w/v) dextrose in water; QC, quality control; LLQ, lower limit of quantitation; t, triplet; m, multiplet; s, singlet.

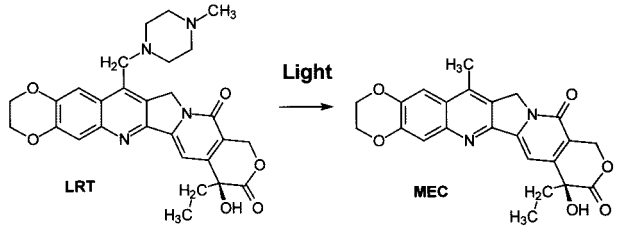

Fig. 1 Chemical structures of lurtotecan and its photodegradant MEC.

Next, the properties of this compound in terms of cell growth inhibition relative to related analogues were examined in addition to the kinetics of lurtotecan photolysis in various aqueous and biological solutions.

MATERIALS AND METHODS

Chemicals. Lurtotecan and NX 211 were supplied by OSI Pharmaceuticals, Inc. (San Dimas, CA). Topotecan was a gift from Smith-Kline Beecham Pharmaceuticals (King of Prussia, PA), and SN-38 was supplied by Aventis (Alfortville, France). RPMI 1640, penicillin, streptomycin, and L-glutamine were purchased from Life Technologies, Inc. (Gaithersburg, MD), and FCS was delivered by Hyclone (Logan, UT). ACN and MeOH, both HPLC-grade, were obtained from Biosolve (Valkenswaard, the Netherlands), and DMSO was from Rathburn (Walkerburn, United Kingdom). Baker (Deventer, the Netherlands) delivered trichloroacetic acid, acetic acid (99–100%), PCA (70%), and HCl (36–38%), and ammonium acetate was from Roth (Karlsruhe, Germany). Sulforhodamine B and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). PBS, obtained from Oxoid and supplied by Boom (Meppel, the Netherlands), consisted of tablets containing sodium chloride (8.0 g/l), potassium chloride (0.2 g/l), disodium hydrogen phosphate (1.15 g/l), and potassium dihydrogen phosphate (0.2 g/l), each dissolved in 100 ml of purified water. All water used in the study was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA).

Isolation and Purification. MEC was prepared by exposure of 17 mg of the lactone form of LRT in a total volume of 40 ml of DMSO-0.1 M ammonium acetate (pH 5.5; 1:4, v/v) to 254-nm UV light in a Beam-Boost photochemical reactor unit (ICT-ASS-Chem, Bad Homburg, Germany) for 5 min. After irradiation, the sample was acidified with 400 μl of PCA and applied onto four C_{18} solid-phase extraction cartridges (Applied Separations, Allentown, PA). Subsequently, the cartridges were washed three times with 1 ml of 25 mM ammonium acetate (pH 3.0), followed by three times with 1-ml volumes of a mixture of 25 mM ammonium acetate (pH 3.0):MeOH (6:4, v/v). The columns were eluted with three 1-ml volumes of 25 mM ammonium acetate (pH 3.0):MeOH (1:9, v/v), followed by three 1-ml volumes of MeOH. The eluate was dried at 60°C under nitrogen until ~1 ml was left over, which resulted in a precipitate in the sample. To resuspend the precipitate, the sample was diluted with 10% PCA:ACN:PBS (2:1:6, v/v/v) to 12 ml, and 200-μl portions of this mixture were injected onto the HPLC system as described previously (11) with the flow rate set at 3.75 ml. The peaks with a retention time of 17 min were collected, in a total volume of ~1 liter, which was acidified with 10 ml of PCA and filled with water up to 2 liters. Further purification was achieved on C_{18} solid-phase extraction cartridges, which were washed three times with 1 ml of water, followed by elution with 5 volumes of 1 ml of MeOH. The eluate fractions were collected and lyophilized using a FDC206 freeze-drying chamber (Savant, Farmingdale, NY) to obtain the pure compound for chemical and biological characterization.

Structural Analysis. The isolated powder was characterized by ^{1}H NMR spectrum, measured on a Bruker AM-300 (300 MHz, FT) spectrometer. Chemical shift values are reported as δ values in ppm using DMSO-D6 (2.5 ppm) as a standard.

Cytotoxicity Assays. The human colon carcinoma WiDr, the ovarian adenocarcinoma IGROV-1, the M-19 melanoma, the H226 lung cancer, the renal cancer cell A498, and the MCF7 and EVSAT breast cancer cell lines were grown and maintained in RPMI 1640. Cells were kept in continuous logarithmic growth at 37°C in a humidified atmosphere in 5% CO_2 in media supplemented with 10% (w/v) heat-inactivated FCS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine. Exponentially grown cells were trypsinized and plated (200 μl with 2000 cells/well) in 96-well culture plates (Costar Corp., Cambridge, MA) 48 h before drug exposure. The stock solution of MEC was diluted in a mixture of DMSO:HCl (98:2, v/v), resulting in an 18 μM concentration, whereas lurtotecan, topotecan, and SN-38 were dissolved separately in DMSO:HCl (98:2, v/v) to obtain 180 μM concentrations. Before addition to the cells, the solutions were diluted 20-fold in culture medium, resulting in 0.90 μM concentrations of MEC and 9.0 μM lurtotecan, topotecan, and SN-38. A 100-μl volume of these solutions was added to the cells, followed by 3-fold serial dilutions in the 96-well plate. Subsequently, the plates were incubated for a period of 5 days. For comparative purposes, MEC, lurtotecan, topotecan, and SN-38 were evaluated in parallel in all experiments. After fixation with 10% (w/v) aqueous trichloroacetic acid, inhibition of cell proliferation was assessed using sulforhodamine B staining as described previously (12), with minor modifications (13). Each compound was tested in quadruplicate in three independent experiments. Cell survival was plotted relative to controls incubated in medium in the absence of the drugs. IC_{50}s were calculated after fitting the individual curves to a Hill function using the software package Siphar version 4.0 (Inna Phase, Philadelphia, PA).

Analytical Methods. Total lurtotecan concentrations in human whole blood were determined using a validated HPLC method (Ref. 10). In brief, aliquots of 50 μl of heparinized whole blood were pretreated with 500 μl of 5% (w/v) aqueous PCA:ACN (5:1, v/v), followed by vigorous vortex-mixing for 30 min. Subsequently, the samples were centrifuged for 5 min at 23,000 × *g*. The HPLC system was identical to that described previously for the determination of lurtotecan concentrations in urine (11), with the flow rate set at 1.25 ml/min. For determination of MEC in whole blood samples, the HPLC system consisted of a constaMetric 3200 solvent delivery system (LDC Analytical, Riviera Beach, FL), a Waters 717plus autosampler (Milford, MA) from which the sample tray was protected from light, an Inertsil-ODS 80A analytical column (150 × 4.6 mm internal diameter; 5-μm particle size; Alltech Applied Sciences, Breda, the Netherlands) maintained at 60°C by a model SpH99 column oven (Spark Holland, Meppel, the Netherlands), and a

Jasco FP-920 fluorescence detector (Jasco, Maarssen, the Netherlands) operating at excitation and emission wavelengths of 378 and 420 nm (40-nm band width), respectively. The mobile phases consisted of 1 M ammonium acetate:water:ACN (1:6:3, v/v/v), with the pH adjusted to 5.5, and the flow rate was set at 0.50 ml/min.

MEC was extracted from 50-μl aliquots of heparinized whole blood using 250 μl of a mixture of MeOH:ACN (1:1, v/v) in 1.5-ml polypropylene tubes (Eppendorf, Hamburg, Germany). The samples were vigorously vortex-mixed for 5 min on a multitube vortex mixer, followed by centrifugation for 5 min at 23,000 × *g* at ambient temperature. A volume of 150 μl of the clear supernatant was transferred to a new 1.5-ml polypropylene tube, followed by addition of 250 μl of 25 mM ammonium acetate (pH 3.0). Subsequently, a volume of 300 μl was transferred to a low-volume insert of glass, from which 200 μl were injected into the HPLC system. The calibration curves were constructed in human heparinized whole blood in concentrations of 0.10, 0.25, 1.00, 2.50, 5.0, and 10.0 ng/ml, by serial dilutions in PBS of a MEC working solution containing 10 μg/ml in DMSO. Four pools of QC samples containing MEC were prepared in heparinized whole blood at concentrations of 0.40, 4.00, 8.00, and 80.0 ng/ml MEC, by addition of appropriate volumes of dilutions of MEC in PBS to whole blood. A fifth QC sample containing 800 ng/ml NX 211 was also prepared in heparinized whole blood to minimize potential differences with clinical samples.

Validation of the assay included a set of calibration samples assayed in duplicate, with LLQ and QC samples in quintuplicate, and was performed on four separate occasions. Within-run and between-run precisions were calculated by one-way ANOVA for each concentration, using the run-day as variable. The extraction recovery for MEC was calculated by comparing peak heights obtained from processed samples containing 2.50 ng/ml MEC in PBS to those obtained in extracted calibration samples.

Stability of NX 211. The increase of MEC in plasma and whole blood containing NX 211 (800 ng/ml) was tested on three separate occasions at ambient temperature, by placing 250-μl aliquots of the samples in the dark and in sodium light (lamp, Philips SON-T-Plus 70 W), laboratory light (lamp, Philips TLD 36W), and in sunlight. After a 2-h incubation, 50-μl aliquots were stored in duplicate at −80°C until analysis. The plasma and whole blood concentrations of MEC were determined using the method described earlier and were compared with MEC concentrations in nonincubated samples. The increase of MEC in the extracts of the QC sample containing NX 211 (800 ng/ml) was tested by placing the extracts on three separate occasions at ambient temperature in laboratory and sodium light. After 1- and 2-h incubation periods, the extracts were placed in the autosampler, and peak heights were compared with those of extracts stored in the dark. The light sensitivity of the clinical formulation of NX 211 was tested by placing serial dilutions of NX 211 in D5W in the dark and in sodium and laboratory light. After 2 h, 50-μl aliquots were stored in duplicate at −80°C and were analyzed for MEC concentrations using the assay described, within 1 week after the experiments. The concentrations of MEC in the incubated samples were compared with nonincubated serial dilutions of NX 211 in D5W.

To rule out a potential metabolic conversion of lurtotecan into MEC, 1-ml aliquots (1 mg/ml) of pooled human liver microsomes (Gentest, Woburn, MA) were incubated in the dark in a phosphate buffer of pH 7.4 at 37°C with 2 μM lurtotecan or NX 211 in the presence of 3 μM NADPH. After a 1-h incubation period, 50-μl aliquots were analyzed for MEC concentrations resulting from metabolic instability, as described for whole blood samples.

Clinical Samples. Whole blood samples for pharmacokinetic analysis were drawn from a vein in the arm opposite to that used for drug infusion from two male cancer patients (ages 39 and 64 years) participating in a Phase I trial (10) and collected in 4.5-ml glass tubes containing lithium heparin as anticoagulant. Samples were obtained before drug administration and at 0.5 (end of infusion), 1, 1.5, 2.5, 4, 6, 8, 24, 48, 72, and 96 h after start of infusion and were immediately transferred to a polypropylene vial and stored at −80°C. The NX 211 was formulated as a sterile liposomal dispersion of lurtotecan in a buffer composed of 10 mM ammonium chloride and 9% sucrose and was supplied in 50-ml clear glass vials containing 5 mg of the active agent. Each vial contained 80 mg of hydrogenated soy phosphatidylcholine, 19 mg of cholesterol, 0.9 g of sucrose, 2 mg of citric acid, and 5 mg of ammonium chloride to a total volume of 10 ml. Dose solutions for administration were prepared in polyvinylchloride-free infusion syringes under aseptic conditions by dilution of the pharmaceutical preparation with D5W to a volume of 25 ml. The solution was delivered by a 30-min i.v. infusion and was protected from light by wrapping the syringe in aluminum foil. Small aliquots of the NX 211 solutions were stored at −80°C for analysis of MEC and total lurtotecan (11) concentrations. The current clinical protocol was approved by the Rotterdam Cancer Institute Review Board, and both patients signed informed consent before study entry.

RESULTS

Structural Identification. The principal unknown peak observed in plasma samples of patients receiving NX 211 (11) was prepared and isolated (0.6–0.7 mg) after irradiation of lurtotecan and dried down to a pale-yellow powdery residue. The purified compound yielded NMR spectra with the aromatic part very similar to that of LRT (Ref. 14) [δ = 0.87, t, 3H, J = 7.2 Hz, 19-CH_3; δ = 1.87, m, 2H, 18-CH_2; δ = 2.67, s, 3H, B-ring CH_3; δ = 4.43, s, 4H, O-CH_2-CH_2-O; δ = 5.22 and δ = 5.42, 2×s, 2×2H, CH_2s 5 and 17; δ = 7.25, s, 1H, Ar-H; δ = 7.54, s, 1H, Ar-H; δ = 7.59, s, 1H, Ar-H]. However, the eight protons of the piperazino group on the B-ring at C-7 at δ = 2.40–2.65 (m) were absent in the isolated product. The signal at δ = 2.67 (s, 3H), characteristic of an *n*-methyl substituent, suggested that scission of the piperazino group had occurred at the N-C bond of the B-ring and thus could be assigned to MEC (Fig. 1). Electrospray ion-trap mass spectrometric analysis also already indicated the loss of the C-7 side chain of the lurtotecan molecule [molecular ion at *m/z* 409 (parent minus C-7 chain)] (Ref. 11). The chromatographic purity of the compound on the reversed-phase system was found to be >99% (data not shown).

***In Vitro* Cytotoxicity.** The cytotoxic properties of MEC relative to lurtotecan and several clinically important analogues were assessed by exposure of each test compound to a panel of

Table 1 Comparative *in vitro* cytotoxicity of MEC and structural analogues

Cell line	MEC IC_{50} (nM)	Lurtotecan IC_{50} (nM)	F[a]	SN-38 IC_{50} (nM)	F	Topotecan IC_{50} (nM)	F
WIDr	2.4 ± 0.035	7.6 ± 1.8	3.1	8.0 ± 0.87	3.3	27 ± 1.9	11
IGROV-1	1.6 ± 0.023	5.4 ± 0.67	3.3	3.5 ± 0.92	2.2	19 ± 2.9	12
M19	25 ± 2.1	80 ± 65	3.2	117 ± 96	4.6	271 ± 185	11
H226	8.1 ± 0.55	15 ± 1.9	1.8	34 ± 40	4.2	103 ± 51	13
A498	3.3 ± 0.17	13 ± 11	4.0	8.5 ± 1.4	2.6	32 ± 7.7	9.7
MCF-7	1.3 ± 0.022	4.3 ± 1.63	3.5	3.5 ± 0.80	2.8	19 ± 4.3	15
EVSAT	8.1 ± 0.58	38 ± 30	4.7	41 ± 31	5.0	147 ± 46	18

[a] F, fold less toxic than MEC.

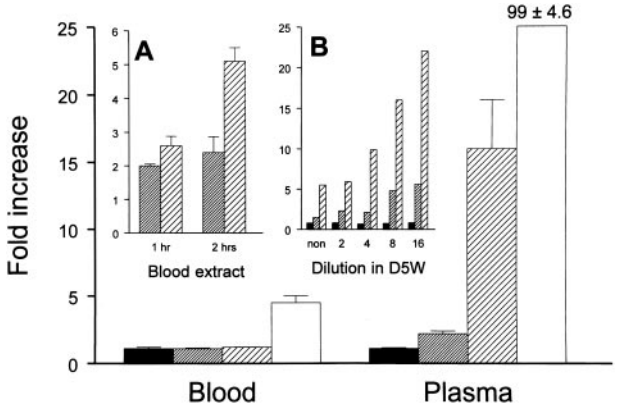

Fig. 2 Stability of lurtotecan in human whole blood and plasma with added NX 211 (800 ng/ml), blood extracts (*inset A*), and aqueous dilutions of NX 211 in D5W (*inset B*) expressed as fold increase in MEC formation relative to a control sample (nominal) after a 2-h incubation period. *Data Represent Means* (*Bars*) ± SD (*Error Bars*). *Columns,* various incubation/light intensity conditions: *black,* darkness; *narrowly hatched,* sodium light; *widely hatched,* laboratory light; *white,* sunlight.

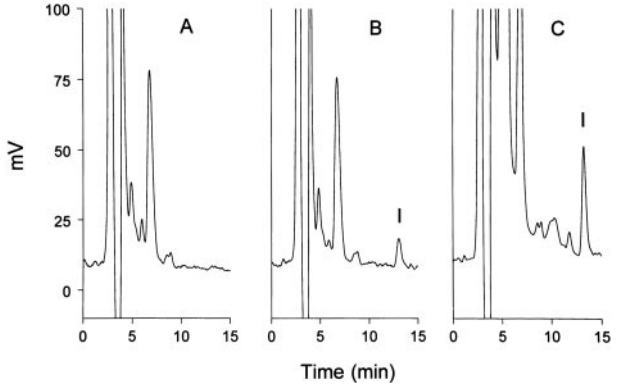

Fig. 3 Reversed-phase HPLC tracings of a blank whole blood extract (*A*), a whole blood sample with added MEC (0.25 ng/ml; *B*), and a whole blood sample with the clinical formulation of NX 211 added (800 ng/ml; *C*). *I,* MEC.

seven cell lines. Under the experimental conditions applied, MEC was found to be a highly potent inhibitor of cell proliferation in all cell lines. Among the tested topoisomerase I inhibitors lurtotecan, topotecan, and SN-38, MEC was found to be the most potent inhibitor of cell growth during a 5-day continuous exposure (Table 1). On average, MEC was 3.4 ± 0.88-fold more potent than lurtotecan, 3.5 ± 1.1-fold more potent than SN-38, and 13 ± 3.0-fold more potent than topotecan in the seven tested cell lines.

Stability of NX 211. To gain insight into the degradation kinetics of lurtotecan as a potential source of MEC formation, experiments were conducted under various conditions and light intensities. In plasma as well as in whole blood, MEC was formed out of NX 211 when incubated for 2 h at ambient temperature in sunlight with, respectively, a 99- and 4.5-fold increase in MEC concentration as compared with samples stored at −80°C (Fig. 2). Plasma samples placed under sodium light and laboratory light also showed increased MEC concentrations, whereas in blood no increase was observed under the same conditions (Fig. 2). An increase in the concentration of MEC also was found after incubation of blood extracts at ambient temperature under sodium light and laboratory light (Fig. 2*A*). Increased concentrations of MEC in the clinical formulation of NX 211 were observed after a 2-h incubation at ambient temperature under sodium and laboratory light. After dilution of this clinical formulation in D5W, the light sensitivity of NX 211 was increased (Fig. 2*B*). In addition, no increases in MEC concentrations were found after a 1-h incubation period of lurtotecan or NX 211 in pooled human liver microsomes, suggesting that the compound was not sensitive to hepatic metabolic degradation.

HPLC Analysis. To obtain information on the pharmacological and toxicological relevance of the lurtotecan photolysis, we set out to develop an analytical method to allow quantitative determination of MEC in samples of patients receiving NX 211 infusions. In view of the relative stability of MEC in whole blood samples, we focused our attention on this matrix to address the pharmacokinetic behavior of the compound. The calibration curves of MEC in human heparinized whole blood were linear over the studied range of 0.10–10 ng/ml, with Pearson's regression coefficients ranging from 0.9986 to 0.9995 using weighted (1/[MEC]) linear least-squares regression analysis. The retention time of MEC was 13 min (overall run time, 15 min), and no endogenous interferences were found in drug-free specimens (Fig. 3). The LLQ of the method was established at 0.10 ng/ml (~0.24 nM), and the within- and between-run precisions of the LLQ and QC samples were ≤7.8%, with the accuracy ranging from 98.4 to 104% (Table 2). The within- and between-run precisions of the QC sample containing 800 ng/ml NX 211 were 9.7 and 17%, respectively. All of the QC samples were found to be stable during

Table 2 Validation characteristics of QC samples for the HPLC assay of MEC

Nominal (ng/ml)	Observed (ng/ml)	WRP[a] (%)	BRP (%)	ACC (%)
0.10[b]	0.10	7.1	2.7	104
0.40	0.40	7.8	[c]	101
4.00	4.01	3.4	6.0	100
8.00	8.23	6.0	[c]	103
80.0	78.7	3.9	7.5	98.4
800 NX 211	0.92	9.7	17	

[a] WRP, within-run precision; BRP, between-run precision; ACC, accuracy.

[b] LLQ samples.

[c] No additional variation was observed as a result of performing the assay in different runs.

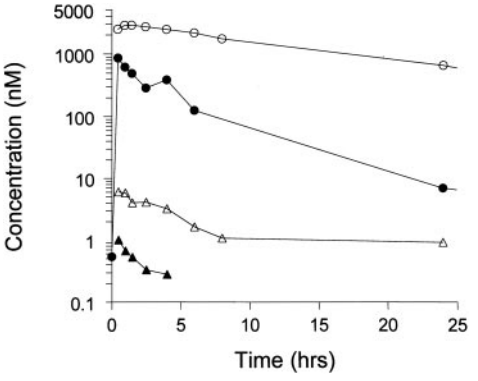

Fig. 4 *In vivo* pharmacokinetics of total lurtotecan (●, ○) and MEC (▲, △) in whole blood of two patients treated with NX 211 [7.3 mg (○, △) and 7.5 mg (●, ▲)] administered as a 30-min i.v. infusion.

three freeze-thaw cycles. The recovery of MEC was constant over the entire range and was 104 ± 11.9% (n = 43). Overall, these validation data of the assay were considered acceptable for its use in clinical studies.

Patient Studies. The two studied patients received 7.5 and 7.3 mg of NX 211 (14.5 and 14.1 μmol), respectively. The total amount of lurtotecan in the infusion solutions of both patients was established at 6.9 mg (13.2 μmol). The total amount of MEC in the sample taken before infusion of patient 1 was 30.1 μg (73.5 nmol), whereas at the end of the infusion the total amount of MEC was increased to 73.5 μg (180 nmol). Thus, the percentage of NX 211 administered as MEC, on a molecular basis, was 0.56% before infusion and 1.4% at the end of the infusion. From patient 2, a sample was taken before infusion, which contained 75.7 μg of MEC (185 nmol), *i.e.,* at least 1.4% of the total amount of drug administered was in the form of MEC. The kinetic profiles of total lurtotecan and MEC obtained from the two patients are shown in Fig. 4.

DISCUSSION

In the present study, we isolated submilligram quantities (0.6–0.7 mg) of a (highly apolar) circulating compound present in the plasma of patients treated with NX 211, a liposomal preparation of the topoisomerase I inhibitor lurtotecan. Following serial purification steps, the product was eventually obtained as a pale yellow powdery residue, and the use of HPLC in combination with fluorescence detection and NMR enabled us to identify its chemical structure. NMR characteristics of the aromatic part were shown to be very similar to that of lurtotecan, and the isolated product was tentatively identified as MEC. This structural assignment is in accordance with our earlier finding of the loss of the C-7 side chain of the lurtotecan molecule using electrospray ion-trap mass spectrometric analysis (11).

MEC was found to be a highly potent inhibitor of cell growth in culture after a 5-day continuous exposure in a panel of seven cell lines, with IC_{50}s on average 3.4-, 3.5-, and 13-fold lower than those of the parent drug lurtotecan, the irinotecan metabolite SN-38, and topotecan, respectively. These data are consistent with earlier findings of the substantially higher *in vitro* cytotoxic activity of 7-methyl-10,11-methylenedioxy-camptothecin compared with 10,11-methylenedioxy-camptothecin after a 3-day continuous exposure (15). The former compound is chemically similar to MEC, with a methylenedioxy substitution at the C10,11 positions in the core structure instead of an ethylenedioxy moiety for MEC. The substitution of the methyl moiety at the B-ring thus resulted in enhanced cytotoxicity, which is in agreement with known structure-activity relationships of camptothecin analogues, indicating that alkylated substitutions at C7 tend to increase topoisomerase I-inhibitory activity and simultaneously decrease aqueous solubility (16). Moreover, 7-chloromethyl-10,11-ethylenedioxy-camptothecin was found recently to be a more potent inhibitor of cell growth than lurtotecan and topotecan in several tumor cell lines during a 48-h continuous exposure (14). In addition, this agent, which has an additional Chloro substitution at the methyl moiety at the B-ring relative to MEC, was almost insoluble in water, which is also in agreement with the current findings.

MEC is most likely a product of lurtotecan photolysis because the parent drug was highly sensitive to light-induced degradation and was not significantly converted to MEC by human liver microsomes. In aqueous solutions, the rate of lurtotecan degradation appeared to be inversely proportional to drug concentration and depended greatly on light intensity, with no noticeable degradation occurring in the dark and progressively increased instability in the presence of sodium light < laboratory light < sunlight. The lurtotecan degradation kinetics in biological fluids was markedly altered, which is most likely associated with stabilizing effects of matrix components, as has been observed previously for anthracycline antineoplastic agents (17). As outlined, such effects might be caused either by increased drug stability attributable to binding to matrix constituents, by quenching of incident light by intense coloring of the sample, or by the presence of particulate matter (*e.g.,* blood cells). In contrast to the photodegradation of lurtotecan, photolysis of irinotecan (CPT-11), one of the most extensively studied camptothecin analogues, only occurs at the fragile lactone-ring structure (18, 19). In view of the close chemical similarity of the lactone moiety between camptothecins, photodegradation of lurtotecan is also likely to occur at this site of the molecule. In general, however, such degradation products are not of major (clinical) interest, because the reactivity of an intact lactone-ring structure is required for the generation of topoisomerase I-

mediated DNA cleavage and antitumor activity of all known camptothecin analogues (20).

The question arises as to whether the additional chromatographic peaks observed in the plasma samples of patients were the result of photochemical degradation during the blood sample handling and subsequent analytical procedure, or that MEC was coadministered simultaneously with the NX 211 infusate. We found that, *in vitro*, the amount of MEC in plasma samples increased 99-, 16-, and 2.2-fold after a 2-h exposure to sunlight, laboratory light, and sodium light, respectively, whereas after incubation in the dark, no increase was observed. In contrast to plasma, MEC concentrations did not increase in whole blood under similar conditions, with the exception of sunlight exposure, where incubation resulted in a 4.5-fold increase in MEC concentration. Although it was beyond the scope of this study to assess the chemical basis of this discrepant behavior, the apparent stability of MEC in whole blood samples provided the possibility to study its disposition in patients. The HPLC method developed for this purpose was shown to be sufficiently accurate and precise to monitor circulating concentrations in patients treated with NX 211. On average, the total amount of MEC in the samples with 800 ng/ml NX 211 added (1544 nM Lurtotecan) was established at 0.92 ng/ml (2.25 nM). Hence, a small fraction (~0.15%) of the clinical formulation of NX 211 is thus already present in the form of the photochemical degradant MEC.

The lower percentages of MEC present in the blood samples of the two studied patients, ranging from 0.061 to 0.24%, compared with values of 0.56–1.4% in the NX 211 infusates, could be indicative for extraliposomal positioning of MEC. The photostability data presented here also suggest that the infusates are far more sensitive to production of MEC than whole blood under laboratory conditions. Currently, we are conducting a retrospective analysis of whole blood samples in a larger group of cancer patients treated with NX 211 to describe in more detail the pharmacokinetic behavior of MEC.

In conclusion, we have shown that lurtotecan in the NX 211 is sensitive to a rapid degradation if exposed to light, resulting in formation of a highly cytotoxic compound that was structurally identified as MEC. The data presented may be of clinical importance because dilutions of NX 211 for use in patients are prepared in aqueous solutions, usually in advance of administrations, as 30-min i.v. infusions. Clearly, the presence of this product at high enough concentrations not only could confound *in vitro* and *in vivo* cytotoxicity and antitumor activity studies but also might distress efforts to elucidate the metabolic fate of lurtotecan and potentially other (new) camptothecin derivatives. Procedures to minimize formation of MEC, by the use of amber vials for NX 211 and by preparation of dilutions immediately before clinical use in a fashion completely protected from any light, are currently being routinely implemented.

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