Metabolism of the new liposomal anticancer drug N4-octadecyl-1-beta-D-arabinofuranosylcytosine in mice

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Originally published at:
Metabolism and excretion of the new antitumor drug N4-octadecyl-1-beta-D-arabinofuranosylcytosine (NOAC) was investigated in mice. Mice were injected i.v. with tritium-labeled liposomal NOAC (4 micromol/mouse). Analysis of HPLC-purified extracts of liver homogenates by liquid chromatography coupled with mass spectrometry revealed only the presence of unmetabolized drug. To study the excretion of the administered drug, mice were injected with tritium-labeled liposomal NOAC or as comparison with 1-beta-D-arabinofuranosylcytosine (ara-C; 4 micromol/mouse) and housed up to 48 h in metabolic cages. Urine and feces were collected at different time points and the kinetics of excreted radioactivity were determined. After 48 h, 39% of the injected [5-3H]NOAC radioactivity was excreted in urine and 16% in feces, whereas ara-C radioactivity was only found in urine with 48% of the injected dose. Feces extracts and urine were purified by HPLC and radioactive fractions were further analyzed by liquid chromatography coupled with mass spectrometry. The radioactivity of feces extracts of NOAC-treated mice was composed of unmetabolized NOAC, hydroxylated NOAC (NOAC + OH), its sulfated derivative (NOAC + OSO3H), and unidentified metabolites, whereas in urine, the hydrophilic molecules ara-C and ara-U were found. During the period of 48 h only 2% of the injected NOAC was eliminated in its unmetabolized form, whereas 25% was identified as main metabolite ara-C. Urine collected during 48 h in ara-C-treated mice contained 33% of the injected dose as unmetabolized drug and 13% as the main metabolite ara-U. Thus, NOAC is metabolized by two major pathways, one leading to the hydrophilic metabolites ara-C and ara-U and the other to hydroxylated and sulfated NOAC.
METABOLISM OF THE NEW LIPOSOMAL ANTICANCER DRUG N⁴-OCTADECYL-1-β-d-ARABINOFLURANOSYLCYTOSINE IN MICE

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(Received August 24, 1998; accepted November 17, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

Metabolism and excretion of the new antitumor drug N⁴-octadecyl-1-β-d-arabinofuranosylcytosine (NOAC) were investigated in mice. Mice were injected i.v. with tritium-labeled liposomal NOAC (4 μmol/mouse). Analysis of HPLC-purified extracts of liver homogenates by liquid chromatography coupled with mass spectrometry revealed only the presence of unmetabolized drug. To study the metabolism of the administered drug, mice were injected with tritium-labeled liposomal NOAC or as comparison with 1-β-d-arabinofuranosylcytosine (ara-C; 4 μmol/mouse) and housed up to 48 h in metabolic cages. Urine and feces were collected at different time points and the kinetics of excreted radioactivity were determined. After 48 h, 39% of the injected [5-3H]NOAC radioactivity was excreted in urine and 16% in feces, whereas ara-C radioactivity was only found in urine with 48% of the injected dose. Feces extracts and urine were purified by HPLC and radioactive fractions were further analyzed by liquid chromatography coupled with mass spectrometry. The radioactivity of feces extracts of NOAC-treated mice was composed of unmetabolized NOAC, hydroxylated NOAC (NOAC + OH), its sulfated derivative (NOAC + OSO₃H), and unidentified metabolites, whereas in urine, the hydrophilic molecules ara-C and ara-U were found. During the period of 48 h only 2% of the injected NOAC was eliminated in its unmetabolized form, whereas 25% was identified as main metabolite ara-C. Urine collected during 48 h in ara-C-treated mice contained 33% of the injected dose as unmetabolized drug and 13% as the main metabolite ara-U. Thus, NOAC is metabolized by two major pathways, one leading to the hydrophilic metabolites ara-C and ara-U and the other to hydroxylated and sulfated NOAC.

Acute myelogenous leukemia can be effectively treated with 1-β-d-arabinofuranosylcytosine (ara-C)¹ (Keating et al., 1982; Gahrton, 1983; Plunkett and Gandhi, 1993). However, its usefulness is limited by its rapid deamination to the biologically inactive metabolite 1-β-d-arabinofuranosyluracil (ara-U) (Ho and Frei, 1971). To protect the drug from deamination, a large number of chemical modifications of ara-C were made in the past (Wempen et al., 1968; Kanai and Ichino, 1974; Rosowsky et al., 1982). Aoshima and coworkers (1976) observed enhanced cytotoxicity after modifying the N⁴-amino group with hexadecyl (C₁₆) to behenoyl (C₂₂) fatty acids, whereas acyl modifications with longer or shorter chains did not influence the drug effect compared with ara-C. Structure-activity relationships were also reported for alkyl-ara-C derivatives (Schwendener et al., 1995b). The alkyl modification of ara-C is not susceptible to hydrolysis and therefore more stable than the acyl compounds (Tsuruo et al., 1982). The new derivative N⁴-octadecyl-1-β-d-arabinofuranocytosine (NOAC; Fig. 6) is insignificantly deaminated to ara-U after incubation with human serum or mouse liver microsomes (Schwendener et al., 1995a). The long-chain alkyl-derivatives of ara-C are highly lipophilic without amphiphilic properties and therefore exert no hemolytic toxicity (Koller-Lucae et al., 1997). This is a further advantage compared with the acyl-derivatives. Pharmacological in vitro studies with N⁴-hexadecyl-1-β-d-arabinofuranocyto sine (NHAC) or NOAC suggest mechanisms of action that are different from ara-C. Cell uptake is nucleoside transporter independent, followed by an ara-C 5'-triphosphate formation, which seems to be too low (2.5- to 150-fold lower as compared with ara-C) to contribute to the cytotoxicity of NHAC. Apoptosis of tumor cells was only induced at NHAC concentrations 20 times higher than observed for ara-C (Horber et al., 1995a,b,c). In human tumor xenografts in nude mice, NOAC showed a significantly better effect than ara-C in various leukemias, and an impressive antitumor activity against different solid tumors (Schwendener et al., 1995a). Other lipophilic ara-C derivatives such as ara-C-5'-diphosphate-β-palmityl-D-thiobutyl alcohol and ara-C-5'-diphospho-L-dipalmitin showed activity against solid tumors (Berdel et al., 1989). Interestingly, NOAC is also active after oral administration (Schwendener et al., 1996) as are the lipophilic ara-C derivatives N⁴-palmityl-1-β-d-arabinofuranocytosine (Ohno et al., 1987).

¹ Abbreviations used are: ara-C, 1-β-d-arabinofuranosylcytosine; ara-U, 1-β-d-arabinofuranosyluracil; NOAC, N⁴-octadecyl-1-β-d-arabinofuranosylcytosine; NHAC, N⁴-hexadecyl-1-β-d-arabinofuranosylcytosine; LC/MS, liquid chromatography coupled with mass spectrometry; ESI, electrospray ionization; THU, tetrahydrothiouracil; RT, retention time; TFA, trifluoroacetic acid; NOAC + OH, hydroxylated N⁴-octadecyl-1-β-d-arabinofuranosylcytosine; NOAC + OSO₃H, NOAC + OH with a sulfate group.
stearyl-1-β-d-arabinofuranosylcytosine (Aoshima et al., 1976), and 1-β-d-arabinofuranosylcytosine-5′-stearylphosphate (Kodama et al., 1989; Ueda et al., 1994). Pharmacokinetic analysis of NOAC in mice gave parameters typical for a lipophilic drug, with distribution into deeper compartments, leading to a prolonged circulation and therefore an elimination half-life of 7 h (Koller-Lucaé et al., 1997). In an ongoing phase I study that we are performing with liposomal NOAC at the University Hospital Zurich, the plasma elimination half-life of NOAC given as i.v. infusion, as determined by HPLC, ranged between 11 to 16 h (Koller-Lucaé et al., 1997). Schleyer et al. (1995) observed a comparable elimination half-life of 9 h for the lipophilic ara-C derivative 1-β-d-arabinofuranosylcytosine-5′-stearylphosphate in humans.

NOAC is a good candidate for cancer therapy with potential advantages compared with the related drug ara-C and to other lipophilic ara-C derivatives. In this study we addressed the question whether NOAC is metabolized by ω- and β-oxidation of the side chain as reported for the lipophilic compounds 1-β-d-arabinofuranosylcytosine-5′-stearylphosphate (Yoshida et al., 1990) and N⁴-behenoyl-1-β-d-arabinofuranosylcytosine (Oh-Ishi et al., 1981), or whether NOAC could also be metabolized to ara-C.

Liver homogenates of mice treated with tritium-labeled NOAC were purified by HPLC and pools containing radioactivity were analyzed by liquid chromatography-mass spectrometry (LC/MS). Furthermore, the excretion kinetics of NOAC or ara-C in urine and feces were analyzed. Unmetabolized drug and metabolites were identified from purified feces extracts and in urine by LC/MS.

**Materials and Methods**

**Chemicals.** NOAC (Fig. 6), NHAC, and N⁴-hexyl-1-β-d-arabinofuranosylcytosine were synthesized as described before (Schott et al., 1994; Schwendener et al., 1995a). Soy phosphatidylincholine was obtained from L. Meyer (Hamburg, Germany). Cholesterol (Fluka AG, Buchs, Switzerland) was recrystallized from methanol. Tetrahydrodridine (THU) was from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Rockville, MD). THU solution was obtained by dissolving 1 mg/ml in PBS (THU/PBS). d-tocopherol, all buffer salts, and other chemicals used were either analytical or HPLC grade and were obtained from Merck (Darmstadt, Germany) Fluka, or Scharlau (Barcelona, Spain). Soluene 350 and Ultima Gold scintillation cocktail were from Packard Instruments (Groningen, the Netherlands). Ara-C and NOAC were tritium labeled (1.11 TBq/mmol [5-³H]ara-C and 0.370 GBq/ mmol [5-³H]NOAC) by Amersham Int. (Little Chalfont, UK).

**Preparation of Liposomes.** Small (80–90 nm) unilamellar liposomes were prepared in PBS (pH 7.4) by sequential filter extrusion as described previously (Koller-Lucaé et al., 1997). Liposome size and homogeneity were determined by laser light scattering (Submicron Particle Sizer model 370; Nicomp, Santa Barbara, CA). The basic composition of the liposomes used was 100 mM soy phosphatidylincholine, 20 mM cholestrol, 0.9 mM d-tocopherol, and 20 mM NOAC. For control experiments, 20 mM ara-C was dissolved in PBS. All preparations were traced labeled with [5-³H]NOAC or [5-³H]ara-C, respectively, sterile filtered (0.2 μm, Schleicher & Schuell, Feldbach, Switzerland), and used within 2 days.

**Liver Metabolism of NOAC.** As outlined in the scheme of Fig. 1, which summarizes the methods used for the analysis of the metabolites, mice (pairs of 2 female ICR, 22 ± 1 g) were injected into the tail vein, each with 4 μmol NOAC in liposomes. After ½, 1, 2, and 4 h, the animals were sacrificed by ether anesthesia. Blood was collected by cardiac puncture and the liver perfused with 10 ml THU/PBS. The livers without the gall bladder were digested as described above. The aqueous and methanolic extracts were analyzed directly, whereas aliquots of the remaining pellet were digested in 2 ml Soluene 350 and neutralized with concentrated hydrochloric acid after scintillation counting. Then the homogenates were ultra-centrifuged (1 h, 100,000g, 10°C, SW 40 Ti rotor, Beckman ultracentrifuge; Beckman Instruments, Fullerton, CA). The supernatant containing the cytosol was removed and the remaining pellet with membranes, nuclei, microsomes, and mitochondria extracted with 6 to 8 ml methanol at room temperature in a bath sonicator (Branson, Danbury, CT) for 1 h followed by centrifugation (10 min, 800g, 20°C). Aliquots of the cytosol and methanolic extracts were counted for tritium activity in a Tri-Carb Liquid Scintillation Analyzer (Packard Instruments). Aliquots of resuspended pellets were digested as described above. As a control, the liver of an untreated mouse was processed identically.

**Urine and Feces.** Groups of three mice (female ICR, 22–26 g) were housed in metabolic cages (Tecniplast, Buguggiate, Italy) with free access to food and water. After a 24-h adaptation, they were injected in the tail vein with a volume of 200 μl containing 4 μmol liposomal NOAC or ara-C dissolved in PBS (see Fig. 1). Three cages were run in parallel. Urine and feces were collected in separate tubes. To prevent deamination of ara-C, 0.5 ml THU/PBS was added to the urine collecting tubes. The tubes were exchanged after the adaptation period and 4, 8, 24, and 48 h after drug injection. The mice were transferred every 24 h into clean metabolic cages to record the differences of food and water intake and amounts of excrements produced before and after treatment. The samples from the adaptation period were used as untreated controls. Before scintillation counting, feces were extracted by sonication for 1 h once in a volume of 10 ml PBS and twice in an equal volume of methanol as described above. The aqueous and methanolic extracts were analyzed separately. Supernatants and urine were measured directly, whereas aliquots of the remaining pellet were digested in 2 ml Soluene 350 and prepared for counting as described before. Values are calculated as percentage of injected radioactive activity. Data were fitted with a hyperbolic equation to calculate saturation.
of all time points, were analyzed with the HPLC method as liver cytosol and the methanolic liver extracts of treated and untreated mice.

The autosampler was equipped with a 92-Varian, Sunnyvale, CA, and a column-heater Croco-cil (Cluzean-infolabo 1995) consisting of a 9010 pump, a 9100 autosampler, a 9050 UV-Vis-detector with a guard column packed with the same material (Phenomenex 30

Metabolites and unmetabolized excreted drug, corresponding fractions were pooled for each extract and cumulated over 48 h. Pools I, II, and III eluted with a binary mixture of 0.05% (v/v) trifluoroacetic acid (TFA) in water (solvent A) and methanol/THU/TFA at 90:10:0.05% (v/v) (solvent B). The mobile phase was initially composed of 90% solvent A and 10% solvent B for 7 min. It was then linearly changed to 100% solvent B within 18 min and held isocratic for 20 min and programmed to return to starting conditions within 5 min. The flow rate was 0.1 ml/min and the injection volume 5 μl (Breithaupt and Schick, 1981). UV absorption was monitored online at 270 nm before entering the MS.

LC/MS. LC/MS samples chromatographed with HPLC method a were dissolved as follows. Urine, feces (pool I), N3-hexyl-1-β-arabinofuranocytosine, ara-U, and ara-C standards in solvent A of HPLC method c. Pool II, pool III, NHAC, and NOAC standards in solvent B of HPLC method c. The samples were dissolved in a volume of 0.25 ml and the reference compounds were dissolved at 0.2 mg/ml. Analyses were performed with HPLC method c on a Platform LC mass spectrometer (Micromass, Manchester, UK). For the detection of negative ions, the mobile phase of the HPLC method c was run without TFA. Samples were monitored under positive (+ESI) or negative (−ESI) electrospray ionization conditions with full scan from m/z 50 to 1000, a scan time of 1 s/scan, and a cone voltage of either 42 or 100 V. The needle potential was set to 4.23 kV. Calibration and sensitivity tests were performed with C54 and NaN4O, for −ESI.

To detect ara-C or ara-U in urine of treated mice the prepared fractions (RF 4 –5 min and 5–8 min) from HPLC method b were dissolved in 0.05 ml (NOAC mice) or 0.25 ml (ara-C mice) ammonium acetate buffer (0.01 M, pH 7). The fraction (RF 4 –5 min) of the THU/PBS extract of feces from a NOAC-treated mouse and controls were treated alike. Ara-C and ara-U standards were dissolved in ammonium acetate buffer to 0.2 mg/ml. The LC/MS system was run using the HPLC system b, which contained no TFA in the mobile phase. Therefore, 1% (v/v) TFA in methanol was added to the eluent after the column at 0.035 ml/min to enhance the formation of positive ions, using a Harvard syringe pump model 22 (Harvard Instruments, Gams, Switzerland) equipped with a gas-tight syringe (Hamilton, Reno, NV). Full-scan +ESI spectra were acquired from m/z 100 to 300 at 1 s/scan, a low cone voltage of 15 V, or a high cone voltage of 42 V, and a needle potential of 3.00 kV. All LC/MS analyses were scanned for mass spectra differing from untreated controls.

Aliquots of 20 μl from all collected fractions were measured for radioactivity. Fractions containing high levels of radioactivity (RF 4–5 min and 5–8 min) were lyophilized and stored at −20°C until LC/MS analysis. The THU/PBS extract of feces from a NOAC-treated mouse was also purified with HPLC method b.

Method c. HPLC method c was operated on a HP Series 1100 instrument (Hewlett Packard Schweiz AG, Urdorf, Switzerland). Chromatography was performed on a 5 column (150 × 1.0 mm; Phenomenex) with a binary mixture of 0.05% (v/v) trifluoroacetic acid (TFA) in water (solvent A) and methanol/THU/TFA at 90:10:0.05% (v/v) (solvent B). The mobile phase was initially composed of 90% solvent A and 10% solvent B for 7 min. It was then linearly changed to 100% solvent B within 18 min and held isocratic for 20 min and programmed to return to starting conditions within 5 min. The flow rate was 0.1 ml/min and the injection volume 5 μl (Breithaupt and Schick, 1981). UV absorption was monitored online at 270 nm before entering the MS.

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Results

Liver. NOAC (4 μmol)-treated mice were sacrificed at different time points. The blood and liver concentration versus time curves (data not shown) were comparable with earlier experiments (Koller-Lucae et al., 1997). Peak concentration in the liver was found to be 35% of the applied radioactivity 1 h after injection. Cytosol was isolated from homogenized livers by ultracentrifugation and the pellet was extracted with methanol as outlined in Fig. 1. The measured ratio of cytosol radioactivity to total liver radioactivity was 1:14 for all time points. Cytosol and methanolic fractions were analyzed by HPLC method a. In all samples, radioactivity was only detected at the R_t of 21 to 23 min, typical for the retention of NOAC. A representative chromatogram of a liver extract from NOAC-treated mice 60 min after i.v. injection is presented in Fig. 2.

Excretion in Urine and Feces. Mice were injected with 4 μmol NOAC or ara-C i.v. and excretion was monitored at different time points (Fig. 3). Toxic effects were not observed after injection of 15% of the LD50 of NOAC (Schwendener et al., 1995a). No difference in volume and weight of excrements, food intake, or body weight between treated and untreated animals was detected when comparing the 24-h collection intervals. As summarized in Table 1, 55 ± 2% of the injected dose per mouse were cumulated after 48 h in total excrements for NOAC and 49 ± 3% for ara-C. The cumulated radioactivity over 48 h gave a ratio between urine and feces of 32:1 for ara-C-treated mice, whereas for animals receiving NOAC, the ratio was 2:1, indicating the distinct differences in the excretion of the two compounds. From the fit of the excretion data presented in Fig. 3, the extrapolated time of quantitative and maximal excretion were calculated. Thus, NOAC radioactivity would excrete to 60% of the injected dose in urine within 43 h and to 54% in feces within 9 days. According to our calculations, the excretion of ara-C should be completed after 12 h. As comparison, Ho and Neil (1997) recovered only 58% of injected radioactivity excreted after 1 week by mice for the lipophilic ara-C derivative ara-C-5'-palmitate. Representative HPLC chromatograms of extracts of feces and urine are shown in Fig. 4A for mice treated with NOAC and in Fig. 4B for ara-C, respectively. From NOAC-treated mice, radioactivity was detected in the void volume with a R_t of 4 to 6 min. Methanolic feces extracts displayed three major tritium-containing peaks eluting as pool I (R_t 4–6 min), pool II (R_t 8–10 min), and pool III (R_t 21–23 min). Pools I, II, and III of the methanolic feces extracts contained UV active (λ = 279 nm) molecules (Fig. 4A). In the urine of ara-C-treated mice, radioactivity was detected only in the void volume with a R_t of 4 to 6 min.

Table 1: Distribution of radioactivity and metabolites identified in HPLC-purified extracts of urine and feces of mice

<table>
<thead>
<tr>
<th>Recovery in excrements (% of Dose)</th>
<th>NOAC</th>
<th>Ara-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC a</td>
<td>39 ± 0.5</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Pool I (min)</td>
<td>4–5</td>
<td>4–5</td>
</tr>
<tr>
<td>Pool II (min)</td>
<td>5–8</td>
<td>5–8</td>
</tr>
<tr>
<td>Pool III (min)</td>
<td>9–11</td>
<td>11–13</td>
</tr>
<tr>
<td>Metabolites</td>
<td>Ara-C</td>
<td>Ara-C</td>
</tr>
<tr>
<td>Ara-U</td>
<td>25 ± 0.3</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>NOAC + OSO_H</td>
<td>11 ± 0.1</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>NOAC + OH</td>
<td>8 ± 0.7</td>
<td>ND</td>
</tr>
<tr>
<td>NOAC</td>
<td>4 ± 0.5</td>
<td>8 ± 0.7</td>
</tr>
</tbody>
</table>

*Four μmol drug per mouse.
*Accumulated over 48 h. Calculated directly from urine or feces extracts. Mean of three experiments.
*HPLC methods are described in Materials and Methods. R_t for retention time.
*Cumulated from pools indicated in Fig. 4. A and B.
*ND, not determined.
*Detected with LC/MS in corresponding pools.

Fig. 4. Representative HPLC chromatograms (method a) of mouse excrements collected between 8 to 24 h after NOAC (A) or 0 to 4 h after ara-C (B) treatment.

In urine of ara-C- or NOAC-treated mice, ara-C and ara-U were identified by LC/MS. NOAC-treated mice contained NOAC in pool I, NOAC + OH in pool II, and NOAC in pool III.
Mice receiving NOAC had the highest excretion rate of radioactivity in all peaks detected by HPLC in urine and feces during the collection interval of 8 to 24 h (Fig. 5). Pool I from feces contained always more radioactivity than pools II or III. The highest ara-C excretion occurred earlier between 0 to 8 h.

**Identification of Metabolites.** From LC/MS spectra of standards it was found that NOAC and related substances like N^4^-hexyl-1-β-D-arabinofuranosylcytosine and NHAC, produced the protonated molecular ion [M+H]^+\), the protonated dimer [2 (M)+H]^+\), and the fragment M-arabinose termed [M'+H]^+\) at a cone voltage of 42 V. By running the standards under collision-activating conditions (cone voltage of 100 V), a signal for the [cytosine+H]^+ moiety appeared in addition to the other signals already detected with a cone voltage of 42 V. Under +ESI conditions, sodium and potassium adducts could be seen occasionally. From chromatography with HPLC method a, it was known that NOAC standard eluted in pool III (R_t 21–23 min). Thus, the HPLC pool III from liver and feces extracts were analyzed by LC/MS (HPLC method c). These samples produced mass spectra identical with those obtained with NOAC standard, with the identical R_t of 33 min. The molecular masses and the spectra of NOAC and its metabolites are shown in Table 2 and Fig. 5. In pool II obtained with HPLC a (R_t of 8–10 min), MS signals differing from blank spectra were detected at a R_t of 32 min with HPLC method c. These mass spectra indicated a mass loss of 132 from m/z 512, which we presume to correspond to a loss of arabinose by rearrangement. The same neutral loss is observed with NOAC (Fig. 5A, m/z 496 to 364). The loss of 18 from m/z 380 to m/z 362 is interpreted as the loss of water and formation of a double bond. We conclude from these results and from the shorter retention time compared with NOAC (LC/MS with HPLC c) that the detected molecule might be hydroxylated NOAC (Fig. 5B, NOAC + OH), with the hydroxyl group located most probably on the alkyl chain (Zsinaï and De Clercq, 1989; Yoshida et al., 1990; Boucher et al., 1996). However, it is not possible to assign the exact position of hydroxylation on the alkyl chain of NOAC. More information about the detected metabolites was obtained by running the LC/MS system with a negative cone voltage. The absence of TFA and formation of a double bond. We conclude from these results and from the shorter retention time compared with NOAC (LC/MS with HPLC method c) allowed us to detect negatively charged molecules. Increasing the pH of the mobile phase resulted in a change of retention times for NOAC (R_t 36 min) and its metabolites (see Table 2). The typical fragmentation patterns [M-H]^−, [M’-H]^−, and [cytosine-H]^− were present again. The expected hydroxylated and sulfated metabolite (NOAC + OSO_3H, Fig. 5C) in pool I (R_t 4–6 min HPLC a) was not detected until running the MS in negative ion mode at a cone voltage of 100 V. The mass spectrum with a R_t of 30 min consisted of [M-H]^−, [M’-H]^−, and a signal of m/z 97 that could be attributed to HSO_3^−, whereas m/z 80 corresponds to the SO_3^−-radical, a marker ion for sulfonates and sulfates (see Table 2). Going back to +ESI conditions, the signal m/z 593 (R_t 28 min) was detected with 42 V but not with 100 V. NOAC (R_t 36 min) and NOAC + OH (R_t 31 min) were also detected by −ESI LC/MS. The comparison of all detected spectra with the corresponding blanks revealed the existence of possible other metabolites in feces pools I and II of HPLC a. However, the signals were not strong enough to draw any conclusions regarding the structure of these metabolites.

### Table 2

<table>
<thead>
<tr>
<th>Pools R_t</th>
<th>LC/MS R_t</th>
<th>Cone Voltage</th>
<th>Molecular Ions</th>
<th>Fragmentation Loss of Arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>min V</td>
<td>[M + H]^+</td>
<td>[M' + H]^+</td>
</tr>
<tr>
<td>NOAC</td>
<td>III (21–23)</td>
<td>33.1 +42</td>
<td>496.5</td>
<td>364.4^c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33.1 +100</td>
<td>496.5</td>
<td>364.4^c</td>
</tr>
<tr>
<td>NOAC + OH</td>
<td>II (8–10)</td>
<td>32.2 +42</td>
<td>512.3</td>
<td>380.4^c</td>
</tr>
<tr>
<td>NOAC + OSO_3H</td>
<td>I (4–6)</td>
<td>28.0 +42</td>
<td>511.9</td>
<td>362.4^c</td>
</tr>
<tr>
<td>NOAC</td>
<td>III (21–23)</td>
<td>35.7 -42</td>
<td>494.5</td>
<td>362.4^c</td>
</tr>
<tr>
<td>NOAC + OH</td>
<td>II (8–10)</td>
<td>31.4 -42^c</td>
<td>510.5</td>
<td>378.9^c</td>
</tr>
<tr>
<td>NOAC + OSO_3H</td>
<td>I (4–6)</td>
<td>29.5 -42</td>
<td>590.5</td>
<td>458.4^c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29.6 -100</td>
<td>590.5</td>
<td>97.0^d</td>
</tr>
</tbody>
</table>

\^a R_t of HPLC method a (for details see Materials and Methods) of feces extract purification.

\^b R_t of MS signal after passage through LC/MS system (method c).

\^c Strongest mass signals are indicated in italic letters.

\^d ND, Not determined.

**Discussion**

Because NOAC is insoluble in aqueous systems, the drug was administered as liposomal formulation. From earlier investigations we found that liposomal NOAC is transferred from the carrier after i.v. application to erythrocytes and plasma proteins, mainly to the lipoproteins. The pharmacokinetic properties of NOAC were assessed with ICR mice, resulting in elimination half-lives of injected radioactivity from blood with 7 h and from the liver with 8 h (Koller-Lucae et al.,
In this study, metabolism and excretion of NOAC were investigated. With the LC/MS analysis of purified liver extracts (without gall bladder) only unmetabolized NOAC was detected at all time points. More hydrophilic metabolites of NOAC were expected to be found in the cytosol or in the methanolic liver extract. Because we could not detect such metabolites, we assume that these metabolites might be cleared from the liver at fast rates after their formation or that they are present at undetectable concentrations. Low amounts of radioactivity were measured in the liver cell cytosol, eluting at the same $R_f$ as standard NOAC. There, the lipophilic NOAC is expected either to bind to proteins, e.g., to the fatty acid binding protein (Storch et al., 1996), or to associate with the lipid droplets. The radioactivity found in feces indicates that NOAC and possible metabolites that were formed in the liver are probably excreted into

![Mass spectra of compounds detected in feces extracts of NOAC-treated mice.](image)

LC/MS analyses were performed with HPLC method c and spectrum A (+ESI, cone voltage 100V), B (+ESI, cone voltage 42 V), and C (−ESI, cone voltage 100V) were recorded from pools III, II, and I of feces extracts purified with HPLC method a. Rearrangements necessary for fragmentation are represented at top of figure. $M^*$ stands for $M\text{-132}$, which is the loss of the arabinose moiety. Peaks in B and C marked with a cross are unidentified compounds.
the gall bladder. This hypothesis is supported by Szinai and De Clercq (1989) who found for a lipophilic deoxyuridine derivative and its metabolites high concentrations in the gall bladder but only low amounts in the liver.

The HPLC chromatogram (Fig. 4) of feces, which gave two peaks different from NOAC and the appearance of 39% of the injected radioactivity in the urine, demonstrates that NOAC is metabolized in the mouse. A model for the metabolic pathway of NOAC is presented in Fig. 6. Modification of the drug occurred within 4 h after application as ara-C, and its typical metabolite ara-U already found in urine in the first collecting interval (Fig. 3). This is in accordance with earlier findings of NOAC radioactivity peaking in the kidneys 3 h after i.v. application (Koller-Luca et al., 1997).

It is conceivable that the formation of ara-C could take place by oxidative dealkylation and cleavage of the C-N bond, producing ara-C and the oxidized alkyl side chain (Testa and Jenner, 1976). As another possibility, the complete β-oxidation of the NOAC alkyl chain in analogy to fatty acid metabolism (Rognstad, 1995) as described for related lipophilic drugs by Yoshida (Yoshida et al., 1990) and Oh-Ishi (Oh-Ishi et al., 1981) could occur. The latter metabolic pathway could be consistent with the finding of the necessary precursor molecule, the hydroxylated NOAC + OH, which was detected in pool II of feces extract eluted by HPLC method a (Rf 8–10 min). Thus, NOAC seems to have several metabolic pathways, because, in addition to the metabolites NOAC + OH and NOAC + OSO3H, the feces extract contained other unidentified MS signals. An obvious pathway to introduce a more hydrophilic group would consist in the sulfatation of the lipophilic metabolites by hydroxylating and sulfatation of the lipophilic alkyl side chain. In future studies we will analyze blood and urine of NOAC-treated human patients for metabolites to elucidate to what extent ara-C might contribute to the antitumor effect of NOAC. Therefore, besides the formation of ara-C, other, still unknown, mechanisms of action might be important.

Summarizing our results, we found that NOAC is metabolized by two major pathways, one leading to the hydrophilic metabolites ara-C and ara-U and one in which NOAC is transformed into more hydrophilic metabolites by hydroxylation and sulfatation of the lipophilic alkyl side chain. In future studies we will analyze blood and urine of NOAC-treated human patients for metabolites to elucidate to what extent ara-C might contribute to the antitumor effect of NOAC.

Acknowledgments. We thank U. Gutteck and R. Bühler for their assistance. We also gratefully acknowledge the Biologisches Zentrallabor of University Hospital Zurich, Switzerland for letting us use their facilities to perform the animal experiments. THU was a generous gift of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Rockville, MD.

### TABLE 3

Summary of retention times and molecular weights of molecules and fragment ions detected in urine of ara-C- (collecting interval 4 to 8 h) or NOAC- (collecting interval 8 to 24 h) treated mice

<table>
<thead>
<tr>
<th>Pools Rf</th>
<th>LCMS Rf</th>
<th>Cone Voltage</th>
<th>Molecule peak</th>
<th>Loss of arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara-C treated mouse:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ara-C</td>
<td>4–5</td>
<td>4.4</td>
<td>15</td>
<td>[M + H]+</td>
</tr>
<tr>
<td>Ara-U</td>
<td>5–8</td>
<td>6.4</td>
<td>15</td>
<td>244</td>
</tr>
<tr>
<td>NOAC treated mouse:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ara-C</td>
<td>4–5</td>
<td>4.6</td>
<td>15</td>
<td>244</td>
</tr>
<tr>
<td>Ara-U</td>
<td>5–8</td>
<td>5.4</td>
<td>15</td>
<td>244</td>
</tr>
</tbody>
</table>

* Rf of fractions prepared with HPLC for LC/MS analysis.
* Rf from LC/MS system run with HPLC method b.
* Strongest mass signals are indicated in italic letters.
Fig. 6. Suggested metabolic pathway of NOAC in mice.

Asterisk indicates position of tritium label.