Lipophilic arabinofuranosyl cytosine derivatives in liposomes

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Abstract

Highly lipophilic drugs can be used therapeutically only by the addition of possibly toxic solubilizing agents or by development of complex pharmaceutical formulations. One way of overcoming these disadvantages is the incorporation of such drugs into the bilayer matrix of phospholipid liposomes. To this end, we chose the approach of chemical transformation of water-soluble nucleosides of known cytotoxic properties into lipophilic drugs or prodrugs. Due to their insolubility, we developed formulations that can be used for intravenous applications in which the lipophilic molecules are incorporated into lipid bilayer membranes of small liposomes. We chose 1-beta-d-arabinofuranosylcytosine (ara-C) as a cytotoxic nucleoside, and we demonstrated that N(4)-acyl derivatives of ara-C were active in vivo in various murine tumor models as liposomal formulations. However, the protection against enzymatic deamination was only partially achieved and was insufficient for significant improvement of cytotoxic properties. Thus, we synthesized a new class of N(4)-alkyl-ara-C derivatives. The most effective derivative, N(4)-octadecyl-ara-C (NOAC), is highly lipophilic and extremely resistant toward deamination. NOAC exerts excellent antitumor activity after oral and parenteral therapy. The activity of NOAC against freshly explanted clonogenic cells from human tumors was determined and compared with conventional antitumor agents. NOAC was used in two liposomal preparations, a stable lyophilized and a freshly prepared liquid formulation. Both formulations inhibited tumor colony formation equally in a concentration-dependent fashion. At optimal conditions, liposomal NOAC had significantly better activity compared with the clinically used drugs cisplatin, doxorubicin, 5-fluorouracil, gemcitabine, mitomycin C, and etoposide. Furthermore, in a hematopoietic stem cell assay, NOAC was less toxic than ara-C and doxorubicin by factors ranging from 2.5 to 200, indicating that this drug is well tolerated at high doses.
Lipophilic Arabinofuranosyl Cytosine Derivatives in Liposomes

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Short title: Lipophilic Ara-C derivatives in liposomes
Introduction

In the field of drug delivery liposomes are predominantly used as carriers for hydrophilic molecules that are entrapped within their aqueous inner volume. Long circulating poly(ethylene glycol) modified liposomes with the cytotoxic drug doxorubicine are an example of such a clinically used formulation. Highly lipophilic drugs can only be used therapeutically by addition of possibly toxic solubilizing agents or by development of complex pharmaceutical formulations. One way of overcoming these disadvantages is the incorporation of such drugs into the bilayer matrix of phospholipid liposomes.

We chose the approach of the chemical transformation of water-soluble nucleosides of known cytotoxic properties into lipophilic drugs or prodrugs. Due to the insolubility of these compounds we developed formulations that can be used for intravenous applications in which the lipophilic molecules are incorporated into lipid bilayer membranes of small unilamellar liposomes. Initially we chose 1-β-D-arabinofuranosylcytosine (ara-C) as cytotoxic nucleoside because the major untoward properties of the drug are the short plasma half-life and rapid degradation by deamination to the inactive metabolite 1-β-D-arabinofuranosyluracil (ara-U), a disadvantage that also impedes the oral application of ara-C.

To abolish these shortcomings a large number of 5′- and N⁴-substituted ara-C derivatives have been synthesized and characterized in the past. We demonstrated that N⁴-acyl-derivatives of ara-C were active in vivo as liposomal formulations at concentrations 2 - 4 times lower than ara-C. However, the protection of the N⁴-acyl-ara-C derivatives against enzymatic deamination was only partially achieved and
suggested to be insufficient for a significant improvement of the cytotoxic properties. Based on these findings, we synthesized a new class of N\(^4\)-alkyl-ara-C derivatives.\(^3,4\) These compounds show a typical structure-activity correlation between the length of the alkyl side chain and their anti-tumor activity.\(^3\) The most effective derivative, N\(^4\)-octadecyl-ara-C (NOAC) is highly lipophilic and extremely resistant towards deamination.\(^5\) NOAC exerts excellent anti-tumor activity after oral and parenteral therapy.\(^6-8\) In a recent study the activity of NOAC against freshly explanted clonogenic cells from human tumors was determined and compared with conventional anti-tumor agents. NOAC was used in two liposomal preparations, a stable lyophilized and a freshly prepared liquid formulation. Both formulations inhibited tumor colony formation equally in a concentration-dependent fashion. At optimal conditions liposomal NOAC had a significantly better activity compared to the clinically used drugs cisplatin, doxorubicin, 5-fluorouracil, gemcitabine, mitomycin C and etoposide. Furthermore, in a hematopoietic stem cell assay NOAC was less toxic than ara-C and doxorubicin by factors ranging from 2.5 to 200, indicating that this drug is well tolerated at high doses.\(^9\) In contrast to the parent drug ara-C, the cellular uptake of NOAC is nucleoside-transporter independent and only insignificant amounts are phosphorylated to ara-C triphosphate (ara-CTP). Furthermore, NOAC is cytotoxic in ara-C resistant HL-60 cells\(^10,11\) and treatment of multidrug resistant tumor cells did not induce P-170 glycoprotein expression, suggesting that the N\(^4\)-alkyl-ara-C derivatives are able to circumvent MDR1 multidrug resistance.\(^12\) We conclude therefore that the mechanisms of action of the N\(^4\)-alkyl-ara-C derivatives are different from ara-C.

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Recently, we further modified NOAC by the synthesis of new duplex drugs that combine the clinically used drugs ara-C and 5-fluorodeoxyuridine (5-FdU) with NOAC yielding the heterodinucleoside phosphates arabinocytidylyl-N⁴-octadecyl-1-β-D-arabinofuranosyl-cytosine (ara-C-NOAC) and 2’-deoxy-5-fluorouridylyl-N⁴-octadecyl-1-β-D-arabinofuransyl-cytosine (5-FdU-NOAC). Depending on the synthesis conditions, the duplex drugs are linked either via 5’→5’ or 3’→5’ bridges. Due to the combination of the effects of both active molecules that can be released in the cells as monomers or as the corresponding monophosphates the cytotoxic activity of the duplex drugs is expected to be more effective as compared to the monomers. Further we anticipate that monophosphorylated ara-C (ara-CMP) and 5-FdU (5-FdU-MP), respectively, are directly formed in the cell after enzymatic cleavage of the duplex drugs. Thus, monophosphorylated molecules would not have to pass the first phosphorylation step, which is known to be rate limiting.

The chemical modification of ara-C and other nucleosides and their formulation in liposomes render these new cytotoxic drugs interesting candidates for further developments.
Materials and Methods

Chemicals

Soy phosphatidylcholine (SPC) was obtained from L. Meyer, Hamburg, Germany. Cholesterol (Fluka AG, Buchs, Switzerland) was re-crystallized from methanol. Poly(ethylene glycol)-dipalmitoylphosphatidyl ethanolamine (PEG-DPPE, Mr 2750) was synthesized as described. DL-α-Tocopherol, all buffer salts and other chemicals used were of analytical grade and obtained from Merck, Darmstadt, Germany or Fluka.

Synthesis of lipophilic arabinofuranosyl cytosine derivatives and heterodinucleoside phosphate duplex drugs

NOAC and all other N⁴-alkyl derivatives of ara-C are synthesized by starting from uridine that is converted to an intermediary 1,2,4-triazolyl compound and reacted with octadecylamine. After cleavage of the O-acetyl protection groups on the arabinose, N⁴-octadecyl-ara-C is obtained as crystalline solid at yields of 80-90%. The heterodinucleoside duplex drugs ara-C-NOAC and 5-FdU-NOAC are synthesized by condensation of NOAC via a 5’→5′ or 3’→5′ phosphodiester linkage to ara-C or 5-FdU, respectively, using the phosphotriester method as described. Figure 1 shows the chemical structures of NOAC and the lipophilic nucleoside duplex drugs.
**Liposome preparation**

A stable lyophilized liposome preparation of NOAC is prepared as follows. The lipids SPC (40 mg/ml), cholesterol (4 mg/ml), D,L-α-Tocopherol (0.2 mg/ml) and NOAC (5 mg/ml) are dissolved in methanol/methylene chloride (1 : 1, v/v) in a round bottom flask. PEG-modified liposomes are obtained by addition of PEG(2000)-DPPE (14 mg/ml) to the basic lipids. The organic solvent is removed on a rotatory evaporator (Büchi, Flawil, Switzerland) at 40°C during 30-60 min. The lipids and NOAC are solubilized by addition of an appropriate volume of phosphate buffer (10 mM, pH 7.4) containing 230 mM mannitol (Fluka, PB-mannitol) that serves as a cryoprotectant, followed by careful shaking of the flask until all lipids are suspended in the aqueous medium. Some glass beads can be added to facilitate the detachment of the lipids from the glass of the flask. Small unilamellar liposomes can be prepared either by high-pressure filter extrusion or by detergent dialysis. In the case of dialysis the detergent sodium cholate (Merck, Darmstadt, Germany) is added initially to the organic lipid mixture at a molar ratio of 0.6 - 0.7 referred to all lipids and NOAC. Alternatively, the detergents octyl glucoside or octanoyl-N-methyl-glucamide (MEGA-8) can be used at a molar ratio of 0.2. After solubilization with PB-mannitol the lipid/drug/detergent mixed micelles are dialyzed against PB-mannitol using a Lipoprep instrument (Diachema, Munich, Germany). Larger volumes (50-500 ml) of liposomes are prepared using a capillary dialysis instrument. The resulting liposomes are sterile filtrated (0.2 µm, Gelman Sciences, Ann Arbor, MI), filled at appropriate volumes (e.g. 10 ml liposomes with 5 mg NOAC/ml) into sterile vials, frozen in liquid nitrogen and lyophilized during 28-48 h (Dura-Dry™ lyophilizer, FTS Systems, New York).
The amount of incorporated NOAC can be determined by addition of a trace amount of tritium-labeled NOAC ($^3$H-NOAC, Amersham Int., Amersham, UK), followed by liquid scintillation counting. Alternatively, NOAC can be quantified by HPLC.$^{17,18}$

The lyophilized liposomes are reconstituted shortly before use with sterile water or 0.9% sodium chloride. Liposome size and homogeneity are monitored by laser light scattering (Submicron Particle Sizer Model 370, Nicomp, Sta. Barbara, CA, USA). Liposome formulations containing the duplex drugs ara-C-NOAC or 5-FdU-NOAC are prepared likewise.

The concentration of NOAC and the duplex drugs ara-C-NOAC or 5-FdU-NOAC in the liposomes can be varied from 1 mg/ml to about 10 mg/ml, depending on the concentration of phospholipids, the lipid composition and the method of liposome preparation. Reconstituted lyophilized liposomes retain their size and homogeneity longer than 72 h after reconstitution. The drugs remain chemically stable during the freeze drying process and reconstitution. Liposome preparations are ready for parenteral use within 1-2 hours after reconstitution.

Properties of NOAC and the duplex drugs

The cytotoxic activity of the nucleoside drugs was tested in vitro on human tumor cell lines using a dye reduction assay.$^{19,20}$ The IC$_{50}$ values for NOAC and the duplex drugs are summarized in Table I. Furthermore, the anti-tumor activity of NOAC and the duplex drugs ara-C-NOAC and 5-FdU-NOAC were confirmed by the National Cancer
Institute (NCI) in vitro drug screening program where they were found to be active against several types of human tumors.\textsuperscript{21}

In an experiment using the L1210 mouse leukemia model we compared the cytotoxic activity of NOAC formulated in plain and in PEG-liposomes of 80-160 nm mean diameter. As summarized in Table II, the antitumor activity of NOAC was excellent, irrespective of the liposome lipid composition. PEG-coating of the liposomes did not improve the anti-tumor effect in the L1210 leukemia. However, it is conceivable that NOAC incorporated in long circulating PEG-liposomes might have improved activity in solid tumors, despite of the transfer of the drug into plasma proteins (see below).

The inhibition of tumor colony forming from freshly explanted human tumors treated with liposomal NOAC is shown in Figure 2 which summarizes the growth inhibitory activity of NOAC after short- and long-term exposure with increasing drug concentrations obtained with lyophilized and freshly prepared liposomes. The lipophilic ara-C derivative had a strong concentration dependent effect on colony formation on a panel of 50 tumor samples of various tumor types. A maximal inhibition of tumor growth was found in 28/50 (56\%) samples with the short-term incubation (1 h) and in 39/50 (78\%) samples with the long-term incubations (21-28 days). At 100 $\mu$M and long-term exposure NOAC proved to be active against tumor cells that were resistant to cisplatin, doxorubicin, 5-fluorouracil, gemcitabine, mitomycin C and etoposide. There was no difference in NOAC activity between the application of lyophilized and freshly prepared liquid liposomes.\textsuperscript{9}

In an in vivo experiment a human acute lymphatic leukemia (ALL) xenotransplanted to NOD/SCID-3 mice was used to evaluate the anti-leukemic effect of NOAC in
comparison to standard agents. As summarized in Table III liposomal NOAC induced the prevention of leukemia development without causing severe side effects at lower doses. The prevention of peritoneal tumors and ascites in the ALL-SCID-3 model was achieved with 8-fold lower drug concentrations of liposomal NOAC as compared to ara-C.\textsuperscript{22}

In several aspects, the lipophilic ara-C derivative NOAC was found to have different pharmacological effects as compared to ara-C. Total uptake after incubation of liposomal NOAC with tumor cells was generally 5-10-fold higher as ara-C. NOAC distributes to more than 90% into the membrane fraction of a cell extract, whereas ara-C is distributed by more than 95% to the cytoplasmic fraction. Furthermore, the uptake mechanism of NOAC was shown to be independent of the nucleoside transport mechanism involved for ara-C and known to be sensitive to dipyridamole and deoxycytidine. NOAC uptake and cytotoxicity were not decreased in the presence of these blocking agents. NOAC is not a substrate for deoxycytidine kinase, the key enzyme of ara-C phosphorylation, suggesting other mechanisms of action.\textsuperscript{5,6,10}

NOAC has several advantages compared to other lipophilic ara-C derivatives such as ara-C ocfosfate (ara-CMP-stearyl ester, YNK-01)\textsuperscript{23} and thioether-linked lipid conjugates\textsuperscript{24} which are orally active prodrugs of ara-C. NOAC is both orally and parenterally active, whereas ara-C ocfosfate and and the thioether-linked lipid conjugates cannot be applied parenterally due to their hemolytic toxicity. Furthermore, NOAC is active against solid and drug resistant tumors, whereas cytarabine ocfosfate and BH-AC are classical prodrugs of ara-C with an activity spectrum that is not
significantly different from the parent drug. In an experimental phase I study with liposomal NOAC the plasma elimination half-life in cancer patients ranged between 11-16 h with peak plasma levels of 180-240 µM at 600 mg NOAC/m^2 without untoward toxic effects, warranting further clinical development of this new drug.\textsuperscript{25}

The duplex drugs were found to be strong inhibitors of the cell cycle. They mainly arrest tumor cells in the early S-phase.\textsuperscript{19,20} With the duplex drugs cell uptake, intracellular distribution and fate and metabolism were not yet investigated. However, due to their similarity to NOAC it can be assumed that they have comparable properties. 5-FdU-NOAC was found overcome 5-FdU resistance in p53 mutated and androgen-independent DU-145 and PC-3 cells.\textsuperscript{20} It is likely that the heteronucleoside dimer is cleaved to 5-FdUMP and NOAC, resulting in sustained intracellular drug concentrations over an extended period, consequently increasing duration and magnitude of the cytotoxic effect. This hypothesis is supported by the fact that the duplex drug specifically inhibits TS activity (see Table I) and that it exerts a cell cycle phase-dependent cytotoxicity, two mechanisms characteristic for 5-FdU. The higher concentrations and longer incubation periods that are required \textit{in vitro} for the cytotoxic effect of 5-FdU-NOAC can possibly be explained by its prodrug nature, resulting in persisting intracellular concentrations of the active metabolites. The slow hydrolysis of the duplex drug by phosphodiesterase-1 and human serum further indicate the prodrug nature of 5-FdU-NOAC. Thus, due to expected changes of the pharmacokinetic properties and the prodrug character of 5-FdU-NOAC the lipophilic duplex drug may have more favourable \textit{in vivo} properties than the individual compounds 5-FdU and

- 10 -
NOAC. In previous studies performed with similar heterodinucleoside phosphate dimers composed of the antivirally active nucleosides azidothymidine (AZT) and dideoxycytidine (ddC) and formulated in liposomes we found significantly different pharmacokinetic properties and superior antiviral effects in comparison to the parent hydrophilic nucleosides in the murine Rauscher leukaemia virus model.\textsuperscript{26}

*Distribution of NOAC to blood cells and plasma proteins*

An important finding was that NOAC is not tightly anchored to the liposomal lipid bilayer and thus is readily distributed in the blood mainly into erythrocyte membranes and lipoproteins.\textsuperscript{27} It is known that unilamellar liposomes aggregate with LDL and allow the transfer of incorporated drugs from the liposomes to LDL. Similar to the observation of others for other lipophilic drugs,\textsuperscript{28} we found that after intravenous injection of liposomal NOAC the drug is transferred within short time from the liposome bilayers to erythrocyte membranes (30%), plasma proteins (<67%) and leukocytes (<2%).\textsuperscript{29}

Thus, liposomes provide an ideal pharmaceutical formulation for NOAC to assure the transfer of the drug to lipoproteins, especially LDL. The natural affinity of NOAC for LDL provides an interesting rationale for the specific delivery of the drug to tumors. Growing and dividing cells require cholesterol for membrane synthesis that is delivered by LDL. This accounts also for cancer cells where an increased LDL uptake in tumors with high metastatic potential and aggressive or undifferentiated character is known. The LDL mediated uptake and cytotoxic effects of NOAC was also studied *in vitro* on Daudi lymphoma cells. NOAC was either incorporated into LDL or liposomes. Specific binding
of NOAC-LDL to Daudi cells was 5 times higher than to human lymphocytes. LDL receptor binding could be blocked and up- or down regulated. In a cytotoxicity test the IC$_{50}$ of NOAC-LDL was about 160 $\mu$M. Blocking the LDL receptors with LDL protected 50% of the cells from NOAC cytotoxicity. The natural affinity of NOAC for LDL provides an interesting rationale for the specific delivery of the drug to tumors that express elevated numbers of LDL receptors.$^{30}$

**Conclusions and prospects**

With the chemical transformation of water-soluble nucleosides into lipophilic compounds and their incorporation into liposomes as their carriers we developed a new class of cytotoxic and antiviral drugs that can be applied for tumor treatment by parenteral routes. Lipophilic ara-C derivatives, particularly NOAC and the duplex drugs composed of NOAC and ara-C or 5-FdU represent very promising new anticancer drugs with high cytotoxic activity and strong apoptosis inducing capability.

Based on our findings we conclude that owing to the chemical modification of ara-C and 5-FdU to molecules that show new physico-chemical properties such as high lipophilicity and stability against enzymatic degradation, together with the possibility to prepare lyophilized liposome formulations of these compounds, more potent drugs are made available. The mechanisms of action of the new drugs seem to be different from those of the parent water-soluble nucleosides, and as an important finding they seem to be able to overcome drug resistance mechanisms. The composition of the liposomes
seems not to represent a crucial factor in the pharmacological properites of the new derivatives. As shown with the L1210 leukemia model, the addition of PEG-lipids did not contribute to an enhancement of the cytotoxic effect (see Table II). This property can be explained with the \textit{in vivo} distribution experiments and the \textit{in vitro} incubation studies with plasma where we observed a fast transfer of NOAC from the liposome membranes to plasma proteins and erythrocytes.$^{27,29}$ Thus, the liposomes serve mainly as a pharmaceutical formulation to permit solubilisation and parenteral administration of the new derivatives.

In conclusion, the chemical modification of water-soluble molecules by attachment of long alkyl chains and their stable incorporation into the bilayer membranes of small unilamellar liposomes represent a very promising method for the development of new drugs not only for the treatment of tumors or infections, but also for many other diseases.

\textbf{Acknowledgements}

The authors thank Daniel Horber, Sibylle Koller-Lucae, Rosanna Cattaneo-Pangrazzi, Iduna Fichtner and Kathrin Friedl for their valuable contributions.
Literature References


Legends to Figures and Tables

Table I
In vitro cytotoxicity (IC$_{50}$) and inhibition of thymidylate synthase (TS) on DU-145 and PC-3 prostate tumor cells by the duplex drugs in comparison to 5-FdU, ara-C and NOAC

Table II
Cytostatic activity of NOAC in plain and in PEG-liposomes in the L1210 mouse leukemia model after intravenous therapy on days 2 and 6.

Table III
Antileukemic activity of liposomal NOAC in the ALL-SCID-3 leukemia model

Figure 1
Chemical structures of NOAC (N$_4^d$-octadecyl-1-β-D-arabinofuranosyl cytosine) and the duplex drugs 5-FdU-NOAC (2'-deoxy-5-fluorouridyl-N$_4^d$-octadecyl-1-β-D-arabinofuranosyl cytosine) and ara-C-NOAC (arabinocytidyl-N$_4^d$-octadecyl-1- β-D-arabinofuranosyl cytosine).

Figure 2
Inhibition of tumor colony forming by NOAC from freshly explanted human tumors
Table I

In vitro cytotoxicity and inhibition of thymidylate synthase

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM) DU-145</th>
<th>50% TS inhibition (µM) DU-145</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM) PC-3</th>
<th>50% TS inhibition (µM) PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FdU</td>
<td>3.4</td>
<td>0.005</td>
<td>3.4</td>
<td>0.006</td>
</tr>
<tr>
<td>5-FdU-(5'→5')-NOAC</td>
<td>4.2</td>
<td>0.66</td>
<td>8.2</td>
<td>0.64</td>
</tr>
<tr>
<td>Ara C</td>
<td>5.4</td>
<td>na</td>
<td>7.3</td>
<td>na</td>
</tr>
<tr>
<td>Ara C-(5'→5')-NOAC</td>
<td>12</td>
<td>na</td>
<td>123</td>
<td>na</td>
</tr>
<tr>
<td>NOAC</td>
<td>134</td>
<td>na</td>
<td>110</td>
<td>na</td>
</tr>
</tbody>
</table>

na, not applicable
Table II

Cytostatic activity of NOAC in plain and in PEG-liposomes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total dose µmol/kg</th>
<th>Survival Time (days) Mean ± S.D.</th>
<th>T/C&lt;sup&gt;b&lt;/sup&gt; %</th>
<th>Survivors 60 days</th>
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</thead>
<tbody>
<tr>
<td>NOAC in PEG liposomes</td>
<td>100</td>
<td>60</td>
<td>857 ± 0</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>52 ± 18</td>
<td>750 ± 18</td>
<td>5/6</td>
</tr>
<tr>
<td>NOAC in plain liposomes</td>
<td>100</td>
<td>60</td>
<td>857 ± 19</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>52 ± 19</td>
<td>744 ± 19</td>
<td>5/6</td>
</tr>
<tr>
<td>Ara-C in PB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>200</td>
<td>20 ± 19</td>
<td>282 ± 19</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>23 ± 20</td>
<td>297 ± 20</td>
<td>1/5</td>
</tr>
<tr>
<td>Controls</td>
<td>--</td>
<td>7</td>
<td>100 ± 0</td>
<td>0/6</td>
</tr>
</tbody>
</table>

<sup>a</sup>, On day 0, 10<sup>5</sup> L1210 cells were injected i.v. into BDF1 mice. I.v. treatment was on days 2 and 6.

<sup>b</sup>, Increase of lifespan T/C% calculated including the 60 d survivors. S.D., standard deviation.

<sup>c</sup>, PB, phosphate buffer (67 mM, pH 7.4)
Table III

Antileukemic activity of liposomal NOAC

<table>
<thead>
<tr>
<th>Substance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Route</th>
<th>Dose/inj.</th>
<th>Peritoneal Tumors</th>
<th>Average tumor burden (ml or g/mouse)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/kg µmol/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment A:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline i.p.</td>
<td></td>
<td>-</td>
<td>6/7</td>
<td>1.8 ± 1.16</td>
</tr>
<tr>
<td>ara-C i.p.</td>
<td>100</td>
<td>411</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>NOAC i.p.</td>
<td>100</td>
<td>200</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>vincristine i.p.</td>
<td>1</td>
<td>1.2</td>
<td>0/7</td>
<td>0</td>
</tr>
<tr>
<td>Experiment B:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline i.p.</td>
<td></td>
<td>-</td>
<td>8/8</td>
<td>2.92 ± 1.69</td>
</tr>
<tr>
<td>NOAC i.p.</td>
<td>50</td>
<td>100</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>NOAC i.p.</td>
<td>25</td>
<td>50</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>NOAC oral</td>
<td>100</td>
<td>200</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>NOAC i.v.</td>
<td>25</td>
<td>50</td>
<td>0/3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Leukemia cells (10^6/mouse) were inoculated i.p. on day zero (6-8 animals/group). Treatment started on day 1. Treatment schedule was on day 1, 4, 7, 11 after tumor inoculation.

<sup>b</sup> Average tumor burden given as sum of peritoneal ascites fluid and solid tumor nodules in ml or gram per mouse.

<sup>c</sup> 3 of 6 mice died due to toxicity.
Figure 1
Figure 2

Inhibitory activity of NOAC

exposure 1 hour  exposure 21-28 days

Inhibitory activity (%)

100
80
60
40
20
0

Liposomes:
- liquid
- lyophilized

NOAC (µM)

1 10 100 1 4 10 100

9/50 17/50 28/50 6/50 15/49 27/50 39/50

treated specimens