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**Title:** Exploring novel formulations and new classes of anticancer drugs in solid tumors  
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Preclinical evaluation and preliminary report of an incomplete phase I pharmacokinetic trial using UNBS1450, a sodium pump antagonist, in patients with advanced solid tumors
ABSTRACT

Introduction
UNBS1450, a semisynthetic cardenolide glycoside derivative is considered a promising anticancer agent targeting overexpressed sodium pump α subunits in malignant tumors. This paper summarizes preclinical data and the preliminary results of a non-completed clinical phase I trial with the compound.

Preclinical data
Experiments on a human hematopoietic cancer cell line, already previously used as cell model to investigate the effects of UNBS1450 were performed in order to evaluate the minimum exposure time to UNBS1450 required to trigger the commitment phase of apoptosis. With this purpose, two strategies were pursued. First, the histiocytic lymphoma U937 cells were incubated for different times with an apoptogenic concentration of UNBS1450 (20 nM) followed by recovery. Second, U937 cells were incubated with 10nM UNBS1450 for 1 h, a concentration/time mimicking the conditions during patients’ treatment. In this instance, the experiment was also performed in presence of different percentages of fetal calf serum (FCS) (0.1-10%) used for cell culture cultivation to monitor any influence of serum to sequester the compound and therefore alter its action. The effect of UNBS1450 on viability (induction of apoptosis) and cell proliferation was then assessed following these conditions during the recovery phase.

Patients and methods used in the clinical trial
A phase I trial to evaluate the safety, tolerability and pharmacokinetics of single agent, UNBS1450 administered once every three weeks in separate cohorts of patients with advanced solid tumors not amenable to established forms of therapy was conducted.

Results of the clinical trial
The study was closed by the sponsor because of bankruptcy before reaching the maximum tolerated dose (MTD) after including 23 patients. The half-life of UNBS1450 was very short being around 0.1 h within the tested dose range. There appeared an approximately linear relationship with dose for both mean maximum plasma drug concentration ($C_{\text{max}}$) and area under the curve 0-t where t is last time at which drug was quantifiable (AUC$_{\text{last}}$) values over the dose range 90-615 µg/patient. There were no Response Evaluation Criteria In Solid Tumors (RECIST) responses in any of the patients.

Conclusion
The primary endpoint of the clinical phase I was not reached due to early termination of the study for non-scientific reasons. The available preclinical work could not guide us in adapted scheduling of the patients. More research is necessary to establish the optimal dose and schedule of UNBS1450 for future phase I/II studies.
INTRODUCTION

The sodium pump, sodium-potassium adenosine triphosphatase (Na+/K+-ATPase), has been suggested as an interesting oncology target. It serves as a versatile signal transducer and it plays a key role in cell adhesion. Several malignancies are characterized by an overexpression of its α subunits.1

Numerous studies investigated the changes in the transmembrane transport of cations during the course of malignant cell transformation, due to increases in Na+/K+-ATPase activity, specifically through the upregulation of the Na+/K+-ATPase α subunits.1 This was confirmed in a large proportion of clinical non-small cell lung carcinoma (NSCLC) samples2 while more than 50% of glioblastoma samples expressed 10 times more α1 messenger ribonucleic acid (mRNA) compared to samples from normal brains.3 Those studies also pointed to a difference in the density of the enzyme, as well as isozyme expression, at the plasma cell membrane of tumor cells.3

Cardiotonic steroids (CSs), and notably cardenolides, are the natural ligands and inhibitors of the Na+/K+-ATPase, thus supporting the possibility of their potential development as anticancer agents targeting overexpressed Na+/K+-ATPase α subunits.4,5 While CSs have been widely used for the treatment of heart failure, early epidemiological evaluations have indicated lower mortality rates in cancer patients who were on digitalis, a cardenolide, at the time of first diagnosis.6-9 To date, the development of CSs as anticancer agents has been impaired by a presumed narrow therapeutic margin resulting from the theoretical risk to induce cardiovascular side effects.1,10

Chemical modifications of 2”-oxovoruscharin (a novel cardenolide extracted from Calotropis procera) based on an understanding of the structure activity relationship within the series, has led to the identification of UNBS1450.11 The activity of the compound in preclinical cancer models, independent of cell type, has been tested in vitro on 57 human cancer models from 11 distinct histological types.11 In aggressive and metastatic orthotopic NSCLC,4,5 refractory prostate cancer12 and glioma3 models, UNBS1450 was more potent than tested reference compounds, including paclitaxel, irinotecan, oxaliplatin, mitoxantrone and temozolomide.3,4,5,12,13

UNBS1450 was the most potent inhibitor of all three isozymes (α3β1, α2β1 and α1β1) with a potency ~6 to > 200 times greater than that ouabain (another cardenolide) and digoxin.3 The general mechanism of action associated with UNBS1450-mediated anticancer effects relates to the compound’s propensity in disorganizing the actin cytoskeleton.3,12,13
UNBS1450 can thus be considered both anti-proliferative (cytotoxic) and anti-migratory given that the actin cytoskeleton is essential to cytokinesis and to cancer cell migration. In sharp contrast to digitalis-like cardenolides, UNBS1450 does not induce intracellular Ca$^{2+}$ or Na$^+$ increase at concentrations at which it induces potent antitumor effects. UNBS1450 induces non-apoptotic cell death processes (such as lysosome membrane permeabilization and autophagy) and thus may overcome major apoptosis resistance pathways responsible in part for the failure of therapeutics in certain cancers. Experimental data involving NF-κB inhibition/deactivation evidenced it as an important new approach to the treatment of various malignancies. UNBS1450 at 10 nM (its mean anti-proliferative IC50 concentration) deactivates the cytoprotective NF-κB pathways at several points, in sharp contrast to specifically designed NF-κB inhibitors acting at one precise point. Furthermore, the anticancer activity of UNBS1450 is not affected by chemotherapy resistance expressed in cancer cells. UNBS1450 kills chemoresistant cells harboring the multidrug resistance phenotype (PgP overexpression) and/or apoptosis-resistant cancer cells with the same efficacy as it does for chemosensitive cancer cells.

Using genomic and proteomic approaches, it was possible to evidence UNBS1450-mediated down-regulation of c-MYC gene, MYC oncoprotein-related genes, and genes with nucleolar functions.

UNBS1450-induced marked down-regulation of c-MYC expression in a number of human cancer cell lines lead to nucleolar disorganization resulting in impairment of cancer cell survival. The present data suggest that c-MYC could be used as a marker of UNBS1450-mediated antitumor activity. An exploratory cardiovascular study in dogs comparing the effects of intravenously administered digoxin and UNBS1450 showed similar effects on the cardiovascular system. There was no evidence of an increased toxicity or increased pro-arrhythmic effects of UNBS1450 compared to digoxin. The structural uniqueness of UNBS1450 taken with its ability to i) disorganize the actin cytoskeleton, ii) disorganize nucleolar structure and functions, iii) kill chemoresistant and/or apoptosis-resistant cancer cells, and iv) deactivate constitutively activated cytoprotective signaling pathways and to induce lysosomal membrane permeabilization and/or autophagy-related cell death thus overcoming major pathways responsible for the failure of cancer chemotherapy, support its development as an anticancer agent targeting overexpressed sodium pump α subunits.

On the basis of the above mentioned presumed antitumor properties of UNBS1450 we started a classical phase I study with the drug.
MATERIAL AND METHODS

Cell culture

U937 cells (histiocytic lymphoma) were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% (v/v) FCS (Lonza, Verviers, Belgium) and 1% (v/v) antibiotic-antimycotic (BioWhittaker, Verviers, Belgium) at 37°C and 5% of CO₂. Experiments were performed in culture medium containing 10% of FCS, unless otherwise indicated. UNBS1450 was a kind gift of Unibioscreen (Brussels, Belgium).

Washout experiments

Cells were seeded at a density of 3.0 × 10⁵ cells/ml and incubated with 20 nM UNBS1450 for different times (0, 1, 2, 4, 6, 8, 10, and 12 h). Then cells were washed and resuspended in the same volume of fresh medium for recovery. As a positive control, cells were cultured in the presence of UNBS1450 throughout the experiment. Apoptosis was analyzed 24-48 h after the start of the treatment (time 0 = T₀).

Determination of apoptosis

a) Analysis of nuclear fragmentation. Percentage of apoptotic cells was quantified as the fraction of apoptotic nuclei (different stages of nuclear fragmentation) assessed by fluorescence microscopy (Leica-DM IRB microscope, Lecuit, Luxembourg) upon staining with the DNA-specific dye Hoechst 33342 (Sigma, Bornem, Belgium). The fraction of cells with nuclear apoptotic morphology was counted (at least 300 cells in at least three independent fields).¹⁶,¹⁷

b) Mitochondrial membrane potential analysis. At the indicated time points, U937 cells were loaded with 50 nM MitoTracker Red (MTR; Molecular Probes)¹⁶,¹⁸ at 37°C for 20 min, and immediately analyzed by flow cytometry using a BD FACScalibur (BD Biosciences, San José, CA, USA), tuned at 488 nm, standard band pass filters FL3 (630 nm). Data were recorded for further analysis with Cell Quest software (http://www.bdbiosciences.com/features/products/display_product.php?keyID=92). The mean fluorescence value was determined by counting at least 10000 cells. Data were further analyzed using FlowJo 8.8.7 software (Tree Star Inc).

c) Induction of apoptosis was molecularly confirmed by Western blot analysis of caspase-3 cleavage (see also section below).
Western Blot analysis

Cells were washed with cold phosphate buffered saline (PBS), and cells extracts were prepared using M-PER® Mammalian Protein Extraction Reagent (Pierce, Erembodegem, Belgium) completed with a protease inhibitor cocktail (Roche, Luxembourg), 1 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 5 mM sodium fluoride (Sigma, Bornem, Belgium). Cells were incubated 15 min at 4°C in lysis buffer and centrifuged at 14000 g, 15 min, 4°C. Twenty μg of proteins were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%), transferred onto polyvinylidene difluoride membranes. Following a 1 h incubation period in 5% non-fat milk in PBS-Tween, membranes were probed with primary antibodies to Mcl-1 and caspase-9 (Cell Signaling Technology, Leiden, The Netherlands; 1:1000 in PBS-T/BSA 5%) and caspase-3 (Santa Cruz, Biotechnology, Boechout, Belgium; 1:1000 in PBS-T/Milk 5%). Protein bands were visualized via chemiluminescence using the ECL+ Western Blotting Detection System Kit® (GE 36 Healthcare, Roosendaal, The Netherlands), following incubation with secondary antibodies horseradish peroxidase (HRP)-conjugated (Mcl-1 and caspase-9: 1:4000, anti-rabbit; caspase-3: 1:4000, anti-mouse; Santa Cruz). Equal loading of samples was controlled using β-actin (Sigma, 1:10000, in PBS-T/Milk 5%; secondary antibody: 1:10000, anti-mouse, in PBS-T/Milk 5%, Santa Cruz).

PATIENTS AND METHODS USED IN THE CLINICAL TRIAL

Study design

This was an open-label, dose escalation study to evaluate the safety, tolerability and pharmacokinetics of single agent, UNBS1450 administered once every three weeks in separate cohorts of patients with advanced solid tumors not amenable to established forms of therapy with curative intent.

The primary objective of this study was to determine the MTD and to establish the recommended phase II dose of UNBS1450 when administered intravenously once every three weeks. The secondary objectives of this study were to describe the safety profile of UNBS1450, including the dose-limiting toxicity (DLT); to assess the pharmacokinetic (PK) profile of UNBS1450; to study preliminary evidence of pharmacodynamic (PD) relationships with UNBS1450 systemic exposure; to perform UNBS1450-related explorative immunomonitoring in peripheral blood; to assess the preliminary antitumor activity of UNBS1450; to study the expression of the Na+/K+-ATPase pump α subunits in the study...
population for potential correlation with clinical responses; to evaluate c-MYC as a potential surrogate marker for antitumor efficacy of UNBS1450 in sequential tumor biopsies.

Dose escalation followed a classical 3 + 3 design. Up to 6 patients were enrolled in this study per dosing cohort. Any patient who received at least 1 dose of UNBS1450 was evaluable for safety. However patients were required to complete at least the initial 21 days of treatment period/observation to be evaluable for determination of MTD, unless they were withdrawn due to a DLT prior to day 22. Patients continued to receive UNBS1450 for as long as the investigator felt it was appropriate but would be discontinued from study drug in case of clinically and/or radiographically documented disease progression; the occurrence of unacceptable toxicity; failure to recover from hematological and/or non-hematological toxicity despite a dosing delay of up to 14 days; medical or ethical reason, including noncompliance and pregnancy (following discussion between the investigator and sponsor); and/or patient's request or investigator's recommendation. Discontinuation of treatment for non-medical reasons (e.g. bankruptcy) was not mentioned in the protocol or contract.

**Eligibility criteria**

The inclusion and exclusion criteria were histologically or cytologically confirmed malignancy that was advanced and/or metastatic and refractory to established forms of therapy or for which no effective therapy exists with curative intent; age 18 years or older; an Eastern Cooperative Oncology Group Performance status (ECOG PS) ≤ 2; left ventricular ejection fraction (LVEF) (by echocardiography) > 55% and no uncontrolled ischemic heart disease; a predicted life expectancy of at least 3 months and adequate hematopoietic, hepatic, cardiac, renal, and thyroid function; no sign of arrhythmias or conduction abnormalities; normal electrolytes; not taking any of the following medication: digoxin, digitoxin or molsidomin, and agents with similar mode of action.

**Study procedures**

Patients received UNBS1450 intravenously once every 3 weeks via central or peripheral intravenous line. The dose was administered over a 1 h infusion, but the actual infusion time could be adapted depending on clinical signs.

Eleven PK samples were collected on day 1 during cycles 1 and 3, between 0 and 5 h after start of infusion (for a 1 h infusion time: prior to dosing, and 20, 40, 60 (just before end of
infusion), 65, 70, 75, 80, 90, 105, and 4 h post start of infusion). Blood samples were taken prior to drug administration on day 1 of cycles 2 and 4.

As this was the first UNBS1450 exposure to humans, the most suitable PD parameters were used during the trial, based on findings made while treating and observing the patients. If possible, the pharmacodynamic biomarker c-MYC and other upstream or downstream markers of the pathway would be assessed in tumor samples and any changes related to PK and clinical outcome.

Blood counts and clinical chemistry (including serum Na⁺, K⁺, Ca²⁺, Mg²⁺, liver function tests, bilirubin, creatinine, blood urea nitrogen (BUN), alkaline phosphatase, total protein, albumin, blood glucose), CBC (complete blood count), and thyroid stimulating hormone (TSH) were obtained at baseline, predose on day 1 (if > 7 days after baseline), and once weekly. Creatine kinase (including MB isoenzyme analysis) and troponin I were also assessed at baseline, predose on day 1, on day 2, and at least weekly on every cycle. Urinalysis were obtained at baseline and repeated predose on day 1 of each cycle. Electrocardiograms (ECGs) were performed at baseline, during infusion, up to 2-4 h post dose on day 1 of each and on day 2 at 24 h post dose, prior to dosing on day 22, and at last study visit. Echocardiography was performed at baseline, on day 22, at end of dosing, and 15 days after last cycle. Echocardiography was assessed by a cardiologist. Physical exams were performed every 3 weeks.

UNBS1450 were supplied in injectable, ready for use, clear glass vials. Each vial contained 10.5 ml (including overfill) with 10 μg UNBS1450/ml of saline solution. The amount of UNBS1450 present per vial was 100 μg. Dosing of UNBS1450 was reduced and/or interrupted for any hematological and non-hematological toxicities related to UNBS1450. Treatment for all patients was repeated provided they reached pre-specified hematological and non-hematological recovery levels (e.g., absolute neutrophil count (ANC) 1.5 × 10⁹/L; platelet count 100 × 10⁹/L; non-hematological toxicity ≤ common toxicity criteria (CTC) grade 1 or ≤ 1 grade worse than baseline severity, etc.). If adequate recovery of these levels was not achieved at time of next dose, dosing was postponed until they were reached. Inpatient dose escalation was not permitted. Dosing was interrupted for any patient with heart rate-corrected QT (QTc) ≥ 470 msec during any ECG. Provided there was no significant cardiac toxicity, dosing might resume at the next lower dose level when QTc has decreased to ≤ 440 msec. Any patient with persistent QTc > 470 msec for more than 1 day (confirmed by a follow-up 10-sec ECG on the next day) was withdrawn. Dosing was stopped if there was any development of clinically significant cardiac arrhythmia or an absolute decrease
of ≥ 10% in the LVEF from baseline. Once a patient’s dose was reduced for a drug-related toxicity, the dose was not re-escalated.

**Criteria for evaluation**

Safety was assessed via physical examination, vital signs, clinical laboratory tests (CBC, clinical chemistry, urinalysis), ECG, echocardiography, and adverse events.

Response assessments (physical examination, CT scan, etc.) were performed every 2 cycles and evaluated according to RECIST version 1.0. Plasma concentration versus time profiles of UNBS1450 was obtained from the analysis of plasma samples. PK parameters were calculated for each subject. Parameters included \( \text{AUC}_{\text{last}} \), area under the curve extrapolated from 0-∞; \( C_{\text{max}} \), %AUC extrapolated, half-life alpha (T\(_{1/2}\) alpha) and T\(_{1/2}\) beta, clearance (Cl), volume of distribution (V\(_z\)) and time of maximum plasma drug concentration (T\(_{\text{max}}\)). A standard 3 + 3 dose phase I dose escalation scheme was used. Pharmacokinetic parameter estimates were summarized by dose cohort using descriptive statistics: N, mean, median, minimum, maximum. In addition, geometric means with 90% confidence intervals were calculated for \( \text{AUC}_{0-\infty} \), \( \text{AUC}_{0-t} \), \( C_{\text{max}} \), drug concentration at 4 h post initiation of drug infusion (C\(_{4h}\)).

**RESULTS OF THE CLINICAL TRIAL**

From October 2008 to October 2010 23 patients were enrolled into seven cohorts in this study in two investigational sites in Belgium and the Netherlands. Patients in cohorts 1 to 7 received single doses of 90, 140, 210, 265, 350, 465 and 615 µg/patient of UNBS1450 as a 60 min i.v. infusion respectively. Two patients, included in the 23 patients, had to be replaced after cycle 1 drug administration owing to non-drug-related adverse events. Additionally, two patients in each of cohorts 2, 3 and 5 and one patient from cohorts 4, 6 and 7 have completed 3 cycles of compound administration (a cycle = one administration every 3 weeks).

In October 2010 the study was closed by the sponsor because of financial reasons. Because of this, MTD was not reached. Not enough data for a response evaluation were available because of the sudden end of the trial.
**PK results**

Given the very short half-life of UNBS1450 determined in the first three cohorts, sampling time points were revised a first time from cohort 4 in order to get more usable data at the early times post administration, and for a second time for cohort 7 where only the last time point during the infusion was changed (from 60 to 55 min). Accordingly, blood samples from cohort 7 were taken predose, at 20, 40 and 55 min during the infusion, and then at 5, 10, 15, 20, 30, 45 and 180 min post end of infusion while for cohorts 1-3, they were taken predose, at 20 and 40 min during the infusion, immediately before the end of the infusion (60 min) and then at 5, 15, 45, 90, 180, 300 and 560 min post infusion. Corresponding plasma samples were analyzed using a validated Liquid Chromatography - Mass Spectrometry and Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) method with a lower limit of quantification (LLOQ) of 0.1 ng/mL at the CRO Notox. As a result of the change in the sampling time points, it was believed more robust PK parameters would have been determined since cohort 4. However, certain calculated PK parameters notably clearance and volume of distribution should still be considered preliminary estimates given the compound's short half-life and inability to follow drug concentrations in plasma generally beyond 0.75 h post end of infusion, despite an appreciably sensitive quantitative method. Furthermore, given the compound's short half-life, $C_{\text{max}}$ and $T_{\text{max}}$ values were likely to be very sensitive to even small errors around sampling times. PK parameters were been determined using a non-compartmental analysis model (Table 9.1).

As explained previously, in this clinical study for the overwhelming majority of patients across all dose groups, $T_{\text{max}}$ has been surprisingly observed earlier than the end of infusion. It had been postulated that this could be due to problems of drug delivery attributable to the infusion pump potentially exacerbated by the extremely short half-life of the compound. Consequently, a change in PK sampling time points was proposed for cohort 7, namely the 60 min post start of infusion sampling time being changed to 55 min, in order to avoid possible sample collection after the infusion had been completed. Unfortunately, this change did not bring the expected outcome, as certainly in two individuals $T_{\text{max}}$ was again observed before the end of infusion at 40 min.

There appeared an approximately linear relationship with dose for both mean $C_{\text{max}}$ and $AUC_{\text{last}}$ values over the dose range 90-615 µg/patient. However, at 465 µg/patient, mean $C_{\text{max}}$ and $AUC_{\text{last}}$ values were lower than might have been expected and only marginally increased over corresponding values determined in cohort 5 dosed at 350 µg/patient (Figure 9.1). Mean $C_{\text{max}}$ and $AUC_{\text{last}}$ values from cohort 7 dosed at 615 µg/patient however
## Summary of PK data obtained after 1 cycle

### Pharmacokinetics of UNBS1450 (mean ± SD, \( t_{\text{max}} \): median (range))

<table>
<thead>
<tr>
<th>Cycle 1</th>
<th>A single dose of 90 μg UNBS1450</th>
<th>A single dose of 140 μg UNBS1450</th>
<th>A single dose of 210 μg UNBS1450</th>
<th>A single dose of 265 μg UNBS1450</th>
<th>A single dose of 350 μg UNBS1450</th>
<th>A single dose of 465 μg UNBS1450</th>
<th>A single dose of 615 μg UNBS1450</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4( a )</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>( C_0 ) (ng/mL)</td>
<td>0 ± -</td>
<td>0 ± -</td>
<td>0 ± -</td>
<td>0 ± -</td>
<td>0 ± -</td>
<td>0 ± -</td>
<td>0 ± -</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (ng/mL)</td>
<td>0.752 ± 0.07305</td>
<td>1.089 ± 0.2972</td>
<td>1.133 ± 0.9001</td>
<td>2.544 ± 0.295</td>
<td>4.211 ± 1.316</td>
<td>4.299 ± 0.9800</td>
<td>8.078 ± 0.6890</td>
</tr>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>0.67 (0.33-0.67)</td>
<td>0.67 (0.67-0.67)</td>
<td>0.33 (0.33-0.67)</td>
<td>0.67 (0.67-0.67)</td>
<td>0.67 (0.67-1.00)</td>
<td>0.67 (0.33-1.00)</td>
<td>0.67 (0.67-1.08)</td>
</tr>
<tr>
<td>( AUC_{\text{last}} ) (ng·h/mL)</td>
<td>0.6492 ± 0.07965</td>
<td>0.8366 ± 0.2462</td>
<td>1.448 ± 0.6409</td>
<td>2.133 ± 0.1093</td>
<td>3.627 ± 1.003</td>
<td>3.886 ± 1.217</td>
<td>5.396 ± 1.133</td>
</tr>
<tr>
<td>( AUC_{\infty} ) (ng·h/mL)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>( \lambda_{\text{alpha}} ) (1/h)</td>
<td>6.751-7.118( a )</td>
<td>4.883( a ) ± 2.435( a )</td>
<td>8.317-9.130( a )</td>
<td>10.21 ± 4.470</td>
<td>7.890( b ) ± 3.999( b )</td>
<td>7.561 ± 2.547</td>
<td>5.812 ± 3.166</td>
</tr>
<tr>
<td>( t_{1/2\alpha} ) (h)</td>
<td>0.09738-0.1027( c )</td>
<td>0.1640( d ) ± 0.06730( d )</td>
<td>0.07592-0.08334( d )</td>
<td>0.0846 ± 0.05313</td>
<td>0.1019( b ) ± 0.04218( b )</td>
<td>0.09994 ± 0.03287</td>
<td>0.1408 ± 0.05979</td>
</tr>
<tr>
<td>( \lambda_{\text{beta}} ) (1/h)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.978( b ) ± 0.3999( b )</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>( t_{1/2\beta} ) (h)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.3616( d ) ± 0.08244( d )</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>( CL ) (L/hr)</td>
<td>125.1-1279( e )</td>
<td>167.3( e ) ± 4765( e )</td>
<td>96.17-169.2( e )</td>
<td>123.5 ± 7.001</td>
<td>100.6 ± 26.65</td>
<td>128.2 ± 40.16</td>
<td>116.6 ± 25.61</td>
</tr>
<tr>
<td>( V_z ) (L)</td>
<td>17.57-18.94( e )</td>
<td>42.20( e ) ± 27.16( e )</td>
<td>10.53-20.35( e )</td>
<td>14.75 ± 8.532</td>
<td>15.50 ± 8.265</td>
<td>18.77 ± 9.597</td>
<td>24.32 ± 13.89</td>
</tr>
<tr>
<td>( CL ) (L/hr)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>117.5( f ) ± 3.822( f )</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>( V_z ) (L)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>60.97( f ) ± 11.73( f )</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Legend:**
- \( C^n \) drug concentration at 4 h post initiation of drug infusion;
- \( C_{\text{max}} \) maximum plasma drug concentration;
- \( T_{\text{max}} \) time of maximum plasma drug concentration;
- \( AUC_{\text{last}} \) area under the curve 0-\( t \) where \( t \) is last time at which drug was quantifiable;
- \( AUC_{\infty} \) area under the curve extrapolated from 0-\( \infty \); \( CL \) total drug clearance;
- \( V_z \) elimination phase volume of distribution;
- \( NA \) not assessable;
- \( 0 = NQ = \) not quantifiable (< 0.100 ng/mL);
- \( * \) calculation based on \( \lambda_{\text{alpha}} \);
- \( b \) calculation based on \( \lambda_{\text{beta}} \);
- \( c \) accurate determination not possible;
- \( e \) \( n = 3 \) for \( \lambda_{\text{beta}}, t_{1/2\beta}, CL_{\text{beta}} \) and \( V_{z\beta} \);
- \( f \) individual values reported. 

\( a = 2, \) individual values reported; \( b = 3, \) accurate determination not possible; \( f = 2, \) individual values reported; \( c = 3, \) individual values reported.
appear to indicate that any previous suggestion that systemic exposure at 465 µg/patient may have plateaued and showed non-linearity was not the case, and was likely due to the limited data forcing the comparison of small size dose groups of different individuals (n = 3 or 4) who have received doses not corrected for body surface area.

However, when dose normalized individual C\text{max} and AUC\text{last} values were compared, there appeared to be a slight trend for a disproportional increase in both these parameters with increasing dose over the range 90-615 µg/patient (Figures 9.2 and 9.3).

**In vitro evaluation of the minimum exposure time to UNBS1450 required to trigger the commitment phase of apoptosis**

To determine the time required for UNBS1450 to trigger the commitment phase of apoptosis, we exposed U937 cells to 20 nM UNBS1450 (a concentration we previously reported as apoptogenic).\textsuperscript{21} Cells were incubated for different times, then, the treatment was washed out and cells were resuspended in fresh medium for recovery. Apoptosis was estimated respectively at 24 and 48 h (as described in Material and methods). Figure 9.4 (panels A-B) shows that a treatment time > 8 h is required to trigger apoptosis, with a more relevant accumulation of apoptotic cells starting from 10-12 h of continuous treatment. The Western
blot analysis of caspase-9 and -3 cleavage further confirmed the results got by estimating apoptosis by two different consolidated approaches, as the analysis of nuclear morphology and fragmentation and the loss of mitochondrial membrane potential (Figure 9.4C). The difference with the positive control was not due to a delay in apoptosis: the same analysis

![Figure 9.2](image1.png)

**Figure 9.2** Dose normalized $C_{\text{max}}$ versus dose group.

![Figure 9.3](image2.png)

**Figure 9.3** Dose normalized $AUC_{\text{last}}$ versus dose group.
performed after 48 h did not show any accumulation of apoptotic cells in samples exposed to washout experiments.

We have identified Mcl-1 protein as the earliest Bcl-2 protein targeted by UNBS1450 in U937 cells.\textsuperscript{21} The findings so far refer to a continuous treatment of the cells with 20 nM

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9_4.png}
\caption{Washout experiments on U937 cells. U937 cells were treated at $T_0$ with 20 nM UNBS1450. Then at the indicated times, the compound was removed and cells were resuspended in fresh medium. At $T_0 + 24$ h and $T_0 + 48$ h, the impact on cell viability of the different times of exposures to UNBS1450 was evaluated by considering (A) the nuclear fragmentation as assessed by Hoechst staining and fluorescence microscope observation; (B) the mitochondrial membrane potential as analyzed by MTR staining and FACS analysis. (C) The induction of apoptosis was further confirmed by caspase-3 cleavage. The results are the mean of three independent experiments or representative of three experiments. Significant difference compared to untreated cells: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.}
\end{figure}

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Next, we explored Mcl-1 protein status during washout experiments with UNBS1450 in U937 cells. The Western blot analysis reported in Figure 9.5 shows Mcl-1 protein levels as estimated after 24 h from the start of treatment with 20 nM UNBS1450 ($T_0$; see also Materials and methods). Mcl-1 appeared down-regulated with the complete disappearance of the protein at times > 12 h.

Since the treatment with UNBS1450 was performed with cell culture medium containing 10% FCS, a percentage which may sequester and, therefore, limit the cytotoxic activity of UNBS1450, we cultured U937 cells in medium with different concentrations of FCS (0.1-10%) in the presence of UNBS1450. Then, U937 cells were cultured again in fresh medium with 10% FCS during the recovery phase. Concentration and time of exposure to UNBS1450 (10 nM; 1 h) were chosen to mimic the concentration used in patients treatment and the turnover of the compound into the body as emerging from the clinical trials. After 24 h and 48 h, the analysis of apoptosis excluded any relevant impact of UNBS1450 on U937 cell viability (data not shown).

Next, we wanted to investigate whether in the same conditions, a cytostatic effect might take place. When the challenge with UNBS1450 was performed in a medium containing 0.1% FCS, we witnessed a significant reduction of the cell concentration in UNBS1450 treated versus untreated cells at 24 h as well as after 48 h of recovery (Figure 9.6A-B). To ascertain whether this reduction effectively corresponded to a reduced cell growth at both time points of recovery, we calculated the 24 h/0 h and 48 h/24 h (of recovery) cell proliferation ratio, which is directly proportional to the doubling time of the cells. The analysis revealed that the cytostatic effect was limited to the early 24 h of recovery, whereas at longer times the rate of cell proliferation was completely restored.

![Figure 9.5](image-url) During washout experiments the decrease of Mcl-1 protein fits with the commitment to apoptosis. U937 cells were treated at $T_0$ with 20 nM UNBS1450. Then at the indicated time points, the compound was removed and cells resuspended in fresh medium. At $T_0 + 24$ h, the impact on Mcl-1 of the different exposures times to UNBS1450 was evaluated by Western blot analysis. The results are representative of three independent experiments with comparable results.
This study was designed to translate preclinical evidence into a clinical phase I study evaluating the safety, tolerability and pharmacokinetics of UNBS1450 administered once every three weeks in separate cohorts of patients with advanced solid tumors or lymphoma not amenable to established forms of therapy with curative intent. The selected route of administration was intravenous because the compound was poorly available when given orally. The intravenous route is also the safest for such a potentially cardiotoxic compound.

**DISCUSSION**

This study was designed to translate preclinical evidence into a clinical phase I study evaluating the safety, tolerability and pharmacokinetics of UNBS1450 administered once every three weeks in separate cohorts of patients with advanced solid tumors or lymphoma not amenable to established forms of therapy with curative intent. The selected route of administration was intravenous because the compound was poorly available when given orally. The intravenous route is also the safest for such a potentially cardiotoxic compound.

**Figure 9.6** Analysis of the impact of FCS on the cytostatic effects of UNBS1450. U937 cells were treated for 1 h with 10 nM UNBS1450 in a medium containing the percentage of FCS indicated in the panels. Then at the indicated times, the compound was removed and cells were resuspended in 10% FCS fresh medium (T₀) for recovery. (A) At T₀ + 24 h (24 h) and T₀ + 48 h (48 h) the cell concentration was estimated by Trypan Blue exclusion assay as described in Material and methods. (B) Cell proliferation index between T₀ + 24 h and T₀ (24 h/T₀; light grey bars); T₀ + 48 h and T₀ + 24 h (48 h/24 h; dark grey bars). The results are the mean of three independent experiments +/- SD. Significant difference compared to untreated cells: *P < 0.05.
as it enabled to perform close cardiac monitoring in each patient who would enter this phase I protocol.

Preclinical pharmacology studies using in vitro and animal models indicate that UNBS1450 is characterized by marked anticancer activity due to both anti-proliferative and anti-migratory (anti-metastatic) features resulting from the propensity of UNBS1450 in disorganizing the actin cytoskeleton, which leads to cell death through autophagy, rather than through apoptosis. In vitro, UNBS1450 kills apoptosis-resistant cancer cells, including multidrug resistant cancer cells. UNBS1450 belongs to the same chemical family as digoxin, a cardiotonic steroid used to treat congestive heart failure. Cardiotonic steroid receptors relate to the α subunits of the sodium pump (the Na⁺/K⁺-ATPase). The α-1 subunit of the sodium pump is overexpressed in 30-40% of a large set of solid cancers, including gliomas, melanomas, renal cell carcinomas, non-small cell lung cancers, and colon cancers. Overexpression of the sodium pump α-1 subunit is also suspected in breast, prostate, and head and neck cancers. The therapeutic ratio with respect to the safety profile/antitumor activity of digoxin is too weak to be used as a potential anticancer agent. On the contrary, UNBS1450 displays tenfold higher binding affinity for the α-1 subunit of the sodium pump than digoxin. With a toxicity profile similar to digoxin, UNBS1450 shows an antitumor activity at least 10 times more pronounced, designating it as a potential candidate for clinical application in oncology, especially where no effective curative therapy exists as it is the case for advanced and/or refractory prostate, breast, non-small cell lung, colon and renal cancers, and for melanomas and glioblastomas. This compound could also find potential use in the treatment of metastatic cancers, knowing that 90% of cancer patients die today from their metastases.

Because of financial reasons this phase I study was closed before reaching the MTD, so it is not possible to establish the recommended phase II dose of UNBS1450. It has to be emphasized again that with such a very short life compound, timing deviations around the sampling time can have a big impact on \( C_{\text{max}}/T_{\text{max}} \) values, while the limited drug concentration-time profile post end of infusion make calculation of accurate pharmacokinetic parameters difficult.

UNBS1450 requires treatment times > 8 h to significantly induce apoptosis in the U937 cancer cell model, when used at the apoptogenic concentration of 20 nM. Mcl-1, which we identified previously as an anti-apoptotic protein early affected by UNBS1450 resulted impacted within the same time required for committing cells to the death. The low concentrations required to affect cancer cells (in the range of nanomolar concentrations) and the fact that UNBS1450 appears to be particularly active on Mcl-1, an intracellular
molecular target currently at the center of many investigations to find out new anticancer therapeutics prompts to explore in the future any further strategies based on the use of this cardiac glycoside in targeting Mcl-1 as potential suitable approach in clinics to fight many forms of cancer.

The percentage FCS does not exert any specific impact on UNBS1450 apoptogenic properties, when used for 1 h at 10 nM. UNBS1450 exerts a cytostatic effect on U937 cells during the first 24 h of recovery when cells are treated in 0.1% FCS medium.

These results may be the base to evaluate specific protocols of administration concerning the number of applications and the lag time between one administration and another, therefore about relevance of tempting different protocols of cycles of treatment alternated to recovery phase.

Moreover, although the absence of a direct impact on cell viability when challenging cells for 1 h with 10 nM of the compound, it would be worth to evaluate whether UNBS1450 may sensitize U937 cells to further cytocidal treatment in combination experiments with known chemotherapeutic agents. Alternatively, it would be considered to evaluate whether UNBS1450 may sensitize U937 cells to further cytostatic treatment in combination experiments with known cytostatic agents. Both assays may provide information about any potential chemoadjuvant activity of this compound, which remains to be determined.

Looking back, we realize that the preclinical work was not extensive enough to start with this phase I trial. Based on washout experiments we think the optimal dose may have been much higher and the optimal schedule more intensively. Because leukemia cells seem to be the most sensitive cells we suggest that a followup phase I study is done in patients with hematological malignancies.

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