Improved Synthesis of N-Benzylaminoferrocene-Based Prodrugs and Evaluation of Their Toxicity and Antileukemic Activity

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ABSTRACT. We have previously demonstrated that N-benzylaminoferrocene-based prodrugs are activated in the presence of the cancer-specific levels of reactive oxygen species (ROS) and are toxic towards several cancer cell lines and primary cells, but non-toxic to representative normal cells (Marzenell, P. et al, J. Med. Chem., 2013, 56(17), 6935; Hagen, H. et al, J. Med. Chem., 2012, 526(2), 924). Herein we report on a substantially improved method of synthesis of these compounds with a yield of up to 90%. We demonstrate its applicability by preparing nine new aminoferrocenes and scaling up successfully the synthesis of the most active prodrug 4 (4-(N-ferrocenyl-N-benzylaminocarbonyloxymethyl)-phenylboronic acid pinacol ester) up to 2 g, which was required for further in vivo experiments reported in this paper. Next, we studied the effects of aminoferrocenes on the viability of selected cancer cell lines (JVM-2, RAJI, BL-2 and HL-60) and primary chronic lymphoid leukemia (CLL) cells having different p53 status (wild type, mutated or completely absent). The obtained data were in agreement with the hypothesis that the toxicity of aminoferrocenes is not dependent upon p53 status. Subsequently the toxicity of prodrug 4 was investigated ex vivo using rat precision cut liver slices (PCLS). Interestingly, prodrug 4 was found to exhibit substantial toxicity (IC_{50} = 3.8 ± 1.3 µM) in freshly prepared PCLS, which initially contained a low amount of glutathione (GSH). In contrast, it was found to be non-toxic up to the highest tested concentration (10 µM) in PCLS pre-incubated for 24 h, that
contained a normal GSH amount. Since this low amount of GSH is likely to correlate with a high intracellular oxidation state, these data are in agreement with the reported mode of activation of aminoferrocenes. In addition, we demonstrated that intraperitoneal administration of a single dose of up to 6 mg/kg of prodrug 4 does not affect the weight and the survival of hybrid male mice BDF1 (DBA/2, ♀ x C57Bl/6, ♂) for at least 21 days after injection. Finally, in a preliminary in vivo experiment prodrug 4 was shown to extend the survival of hybrid male mice BDF1 carrying L1210 leukemia from 13.7 ± 0.6 days to 17.5 ± 0.7 days when injected daily for 6 times at a dose of 26 µg/kg (cumulative dose: 0.156 mg/kg) starting from the second day after injection of L1210 cells. We confirmed that the antitumor activity of prodrug 4 correlates with the increase of the oxidative stress in L1210 cells isolated from treated animals, which is reflected in the higher intracellular ROS concentration and damage of the cellular membrane. All together our data indicate that aminoferrocene-based prodrugs can potentially be applied for the treatment of cancers, which express mutated or no p53 and are often poorly responsive to the conventional chemotherapy.

INTRODUCTION

The intracellular milieu of cancer cells differs substantially from that of normal ones. This difference can be utilized to design cancer specific, ROS-sensitive prodrugs. In particular, the concentration of reactive oxygen species (ROS= ¹O₂, O₂⁻, H₂O₂, HO•) is higher in the majority of cancer cells. It has been suggested that this biochemical alteration is required to support the cancerous phenotype. Therefore, designing prodrugs that need activation by ROS can lead to anti-cancer drugs that are active against a broad range of cancers. Known prodrugs activated at elevated ROS concentrations include the naturally occurring cyclic glycopeptide bleomycin,
hydroxyferrocifen and its analogues, organochalcogene-based pro-catalysts, $H_2O_2$-responsive pro-alkylation agents, SN-38 (a camptothecin derivative) and aminoferrocene-based prodrugs. In particular, bleomycin coordinates intracellular iron ions (present in excess in cancer cells) resulting in the formation of a potent catalyst for cleavage of genomic DNA. Hydroxyferrocifens and organochalcogenes are oxidized to glutathione (GSH) scavengers and ROS-generating catalysts, respectively; thus, inducing cell death via oxidative stress. Furthermore, pro-alkylating agents, such as SN-38 and aminoferrocenes are activated by the cleavage of a C-B bond in the presence of cancer-specific $H_2O_2$ concentrations. In the case of aminoferrocenes two toxic products are obtained, one of them being a ROS-generating catalyst and the other a GSH scavenger. These products act synergistically on each other leading to a strong elevation of ROS concentration in cancer cells causing their death. The dual mode of action of aminoferrocenes is unique. The only other drug reported up to date, acting by a related mechanism, is $[(\eta^6\text{-arene})Ru(azpy)I]^+$. In particular, this compound first oxidizes GSH to GSSG. Then, the reduced complex is oxidized by $O_2$ to give the initial complex and $H_2O_2$. However, in contrast to aminoferrocenes this compound is not a prodrug. Therefore, it can potentially affect also normal cells. Moreover, the oxidized product (GSSG) can be recovered by intracellular reductases thereby diminishing the effect of the drug. In contrast, products released from aminoferrocenes irreversibly modify glutathione by alkylating it.

In previous work we have demonstrated that selected aminoferrocenes are active against primary chronic lymphoid leukemia (CLL) cells. However, in this preliminary study the genetic status of CLL cells was not determined. Nevertheless, it is well documented that CLL is a genetically heterogeneous disease and some CLL-types exhibit poor prognosis. For example, TP53-mutated CLL is poorly responsive to standard therapy. The TP53 gene encodes tumor
suppressor p53, a protein participating in cell cycle regulation. Therefore, search for new drugs for the treatment of TP53-mutated CLL and other hematological cancers is warranted. Since the mode of action of aminoferrocenes relies on ROS-induced chemical activation that is not directly related to TP53, it is probable that these prodrugs will be active against both wild type and TP53 mutated/negative hematological cancers. Herein, we discuss a new series of data proving this hypothesis using three B-cell derived cancer cell lines with different TP53-status and primary CLL cells with defined TP53 status. Moreover, we report on studies of toxicity and antitumor activity of a selected aminoferrocene (compound 4, Scheme 1) using rat precision cut liver slices (ex vivo), and BDF1 (DBA/2, x C57Bl/6, DBA/2, x C57Bl/6) mice carrying TP53-mutated L1210 leukemia (in vivo). For these biological experiments a large amount of prodrug 4 (up to 2 g) was required, which was obtained using a substantially improved method of synthesis of N-benzylated aminoferrocenes. We also demonstrated the general applicability of this method by preparing nine new N-substituted aminoferrocenes.

RESULTS AND DISCUSSION

Synthesis of N-alkylated aminoferrocene-based prodrugs

The previously reported procedure for preparation of compound 4 yielded under optimized conditions only ~5% of the product. It consisted of reductive amination of commercially available aminoferrocene 1 in the presence of benzaldehyde and Na[B(CN)H$_3$] with formation of N-benzylaminoferrocene 2, followed by coupling of compound 2 to 4-(hydroxymethyl)phenylboronic acid pinacol ester in toluene in the presence of triphosgene at 120 °C for 24 h (steps a, b in Scheme 1). Since the intermediate product 2 is unstable in air, these two
Scheme 1. Outline of tested pathways for optimization of synthesis of N-alkylated aminoferrrocene-based prodrugs

\[ \text{RR'C=O, NaB(CN)H}_3; \]
\[ \text{b: 4-hydroxymethylphenylboronic acid pinacol ester, Cl}_3\text{COC(O)OCCl}_3, 120 \degree \text{C}; c: same as b; d: benzyl chloride, Cs}_2\text{CO}_3, \text{tert-butylammonium iodide (TBAI); e: 1,2-di-(chloromethyl)benzol, Cs}_2\text{CO}_3, \text{TBAI; f: compound 3, Cs}_2\text{CO}_3, \text{TBAI.} \]

steps were conducted in a one pot reaction. We observed that changes of conditions including (1) optimization of the reductive amination combined with isolation of compound 2, (2) replacement of triphosgene either for N,N'-carbonyldiimidazole (CDI) or CDI/CH\(_3\)I mixture or 4-nitrophenyloxycarbonylchloride and (3) increasing nucleophilicity of the hydroxyl group of 4-hydroxymethylphenylboronic acid pinacol ester by its deprotonation on the presence of NaH led to further reduction of the yield of the desired product. Moreover, application of substituted benzaldehydes in this reaction sequence with an attempt to prepare analogues of compound 4 was hampered by even lower yields. The latter effect was especially pronounced for pyridinecarboxaldehydes and 2- and 4-substituted benzaldehydes carrying either electron donating substituents like OMe, NMe\(_2\) or CH\(_3\) or thermally and hydrolytically unstable
substituents like OAc or NHAc. Therefore, an alternative approach for synthesis of compound 4 was developed. In particular, we used compound 3 as a starting material. In contrast to 2, compound 3 is highly stable and can be obtained from commercially available starting materials in large quantities with 74% yield.\(^9\) We attempted alkylation of compound 3 (1) under optimized Mitsunobu conditions for carbamates,\(^1\) using benzyl alcohol, Bu\(_3\)P and 1,1'-(azidodicarbonyl)dipiperidine (ADDP); (2) in the presence of Cs\(_2\)CO\(_3\) using BnCl/(NBu\(_4\))I mixture as an electrophile under conditions of Salvatore et al;\(^13\) and (3) in the presence of NaH using either benzyl chloride or bromide as electrophiles as described elsewhere for other starting materials.\(^14\) Though the first approach delivered only traces of the product, the other two reaction conditions led to clean conversion of compound 3 into 4. Isolated yields of analytically pure compound 4 varied in the range between 71 and 92%, which is substantially improved with respect to the previously reported synthetic protocol (5%). We confirmed that this method is suitable for scaling up the synthesis of compound 4 up to 2 g. We also used this protocol to prepare nine new analogues of 4, including compounds, containing differently substituted phenylmethyl and pyridylmethyl moieties. In particular, in the series of compounds 4, 5, 6 electron deficiency of the Ph-rest is modulated by substituents in the 3\(^{rd}\) position: H, F, OMe. In compound 7 the para-hydroxy group is protected as an acetic acid ester. In cells it is expected to be deprotected via hydrolysis forming the highly electron rich 4-hydroxyphenylmethyl-containing aminoferrocene, which will be partially deprotonated at pH 7 with formation of the phenolate ion (pK\(_a\) ~ 9-10). In contrast, 2- and 3-pyridyl-residues in compounds 8 and 9 are strongly electron deficient. At pH 7 they are expected to remain neutral (pK\(_a\)~ 5-6). Compound 10 is derived from 4 by substitution of a benzyl for a diphenylmethyl residue. It is obtained by alkylation of intermediate 3 with diphenylmethylchloride with the relatively low yield of 6%.
The latter result reflects the low reactivity of the corresponding electrophile due to its steric bulkiness. Compound 10 is the first example of the aminoferrocene-based prodrug where the carbon atom connected to the nitrogen atom of the aminoferrocene core carries two substituents (phenyl residues in this case). Synthesis of such derivatives using the initially applied protocol was not possible since the reductive amination product formed from aminoferrocene 1 and ketones in step a is not reactive in the following step b (Scheme 1). Since all steps in the new protocol were conducted at 22 °C, thermally unstable or reactive substrates could be applied. For example, compound 11, which carries 2-chlormethyl residue could be prepared from equimolar mixture of compound 3 and 1,2-di(chlormethyl)benzene. The product was isolated with the yield of 34%. X-ray single crystal diffraction analysis allowed to determine the structure of compound 11 (Figure 1), which is the first crystallographically characterized aminoferrocene-based prodrug. These data confirm the atom connectivity suggested based on the synthesis, $^1$H NMR spectroscopy and mass spectrometry of 11. The geometry of a ferrocene unit in compound 11 is similar to that in other known alkyloxy carbonylaminoferrocenes. Atoms of a carbamate group

![Figure 1](image-url). Molecular structure of compound 11 as determined by X-ray crystallography.
(C2, N1, C3, O1, O2) are located in a plane, which is inclined by 24° to the plane of the
cyclopentadienyl ligand containing atoms C42, C41 and C45 (Figure 1). These data are
indicative of only weak conjugation between the ferrocene unit and the carbamate fragment. The
fully planar conformation is not realized due to the expected clash between the bulky substituents
at N1 atom with the hydrogen atoms attached to C42 and C45 atoms. It is evident that a fragment
–H2C1-C11 is not hindered by other atoms in structure 11 (Figure 1). Therefore, we expected that
it is accessible to nucleophilic reagents. In agreement with this expectation, we observed that 11
reacts with excess 3 with formation of doubly substituted product 12. We are currently exploring
11 for conjugation of the aminoferrocene core to other nucleophilic modifiers, e.g. those, which
can either target these prodrugs to specific compartments in cells (e.g. nuclei or mitochondria) or
direct them to cancer cells. Analogously to mono-aminoferrocene-based compound 3, 1,1’-bis-
aminoferrocene-based compound 13 can be efficiently alkylated at the optimized conditions
producing doubly N-benzylated product 14 with the yield of 55 % (Scheme 2). In this case the
yield is slightly lower than that observed in the alkylation of compound 3 – 71-92 %.
Thus, the synthetic method described above expands our possibilities in the modification of the
N-substituent in aminoferrocenes at the sites indicated with red arrows in Scheme 1, whereas the
previously used protocol allowed only modifications at the sites indicated with blow arrows and
was restricted to thermally stable substrates. Moreover, it allows scaling-up the synthesis of N-
substituted aminoferrocenes that enables their thorough structural, spectroscopic and biological
studies.
Scheme 2. Synthesis of doubly-modified aminoferrocenes using the developed improved synthetic protocol and structures of controls.

\[
\begin{align*}
&\text{controls} \\
&\begin{array}{c}
\text{Fe} \\
15 \\
\text{Fe} \\
16 \\
\text{FeCl}_3 / \text{N} \\
\text{OH} \\
1 \text{eq} / 2 \text{eq}: 17
\end{array}
\end{align*}
\]

a: BnCl, Cs\textsubscript{2}CO\textsubscript{3}, TBAI.

Effects of prepared prodrugs on the viability of B-cell-derived cancer cell lines having different TP53-status

To explore the role of p53 in the mode of action of aminoferrocene-based prodrugs we studied their effects on the viability of three selected B-cell derived cancer cell lines with different TP53 status. The human mantle cell lymphoma (MCL) was represented by the JVM-2 cell line. MCL accounts for ~7% of all lymphoid malignancies and has relatively poor prognosis due to almost invariable relapse occurring after initial chemotherapy.\textsuperscript{16} The JVM-2 cell line contains wild type (wt) p53 and an additional t(11;14) translocation. The human Burkitt lymphoma was represented in our panel by RAJI and BL-2 cell lines. Notably, mutated p53 is expressed in RAJI, whereas wt p53 in BL-2 cells. Moreover, in RAJI a translocation t(8,14) as well as Epstein-Barr-Virus (EBV+) are detected, whereas in BL-2 a translocation t(8,22) is present. The latter cell line is EBV negative.\textsuperscript{17} The promyelocytic human leukemia cell line HL-60, which expresses no
detectable p53 protein and p53 mRNA and whose TP53 gene underwent major deletions,\(^\text{18}\) was also included for comparison (Table 1).

First, we determined the relative amounts of intracellular reactive oxygen species in JVM-2, RAJI and BL-2 by using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA)-based assay as described earlier.\(^\text{9}\) In particular, we observed that the relative mean fluorescence (F) of JVM-2 (F= 0.71 ± 0.01) or BL-2 cells (F= 0.77 ± 0.03) measured after their incubation with DCFH-DA for 30 min was comparable to that of HL-60 cells (F=1.0). In contrast, in these conditions the relative mean fluorescence of RAJI cells was ~3 fold higher than that of HL-60 cells. These data indicate that ROS amounts in JVM-2 and BL-2 cells are similar to that in HL-60 cells, whereas in RAJI cells it is higher than that in HL-60 cells. Since the prodrugs are efficiently activated in HL-60 cells due to their reaction with ROS,\(^\text{9}\) these data indicate that an analogous activation should also be possible in JVM-2, BL-2 and RAJI cells provided that the prodrugs are taken up into the cells.

The physiological concentration of boron-containing compounds in cells is negligible. Since each molecule of the prodrug contains one boron atom, we could investigate the membrane permeability of these compounds by monitoring the boron content in the cells.\(^\text{9}\) Intracellular boron was determined for all four studied cell lines by the curcumin assay in combination with UV-visible spectrophotometry for the representative compound 4. In this assay the uptake correlates with the baseline corrected absorbance at 550 nm (A(550 nm) – A(780 nm)) characteristic for the curcumin-boron complex. In particular, untreated cells give rise to A(550 nm) – A(780 nm) in the range of 0.34-0.38, whereas cells incubated with compound 4 give rise to A(550 nm) – A(780 nm) between 1.30 and 2.36. When the uptake of compound 4 in HL-60 cells is taken as a reference (1.0), the permeability in RAJI cell line
Table 1. Effects of the aminoferrocene-based prodrugs on the viability of the selected cell lines (IC$_{50}$ values) with different p53-status after 48 h incubation.$^i$

<table>
<thead>
<tr>
<th>Drug$^{ii}$</th>
<th>IC$_{50}$ (µM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HL-60 no p53</td>
</tr>
<tr>
<td>3</td>
<td>&gt;50</td>
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<tr>
<td>4</td>
<td>11 ± 1</td>
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<tr>
<td>5</td>
<td>9 ± 7</td>
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<tr>
<td>6</td>
<td>17 ± 4</td>
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<tr>
<td>7</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
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<tr>
<td>10</td>
<td>15 ± 2</td>
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<tr>
<td>11</td>
<td>6 ± 0</td>
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<td>12</td>
<td>~50</td>
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<tr>
<td>13</td>
<td>8 ± 8</td>
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<td>14</td>
<td>10 ± 0</td>
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<td>15</td>
<td>&gt;50</td>
</tr>
<tr>
<td>16</td>
<td>&gt;50</td>
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<tr>
<td>17</td>
<td>3 ± 1</td>
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</tbody>
</table>

$^i$ no p53 – wt p53 is not expressed; mut p53 – mutated p53; wt p53 – active (wild type) p53 is expressed. Data are given as mean of at least three independent experiments ± SD. ND – not determined.

$^{ii}$ Structure of prodrugs and their labeling scheme are given in Schemes 1 and 2.
can be determined to be equal to 1.8 ± 0.5, that in JVM-2 cell line to be 1.5 ± 0.1 and that in BL-2 cell line 1.4 ± 0.1. These data indicate that, within the experimental error, compound 4 permeates equally well through the membranes of all studied cell lines.

Furthermore, we studied the viability of three selected B-cell derived cancer cell lines and of HL-60 cell line in the presence of known (3, 4, 13) and newly prepared aminoferrocene-based prodrugs by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Table 1). Among the new aminoferrocenes reported in this paper, only compound 11, which contains a 2-CH₂Cl residue in the N-benzyl substituent, turned out to be substantially more toxic towards all cell lines tested than the parent compound 4 (p < 0.01). We can speculate that in this case the stronger cytotoxic effect could be caused by alkylation of endogenous nucleophilic moieties, e.g. thiol groups of GSH or proteins, by the 2-CH₂Cl residue. At this point other possible mechanisms, e.g. enhanced uptake of this compound, cannot be excluded. Since the experiments with the CLL cells (as described below) were performed with medium containing human serum (HS), whereas the cell lines were incubated in medium with fetal calf serum (FCS), we investigated whether the replacement of FCS by HS in experiments with HL-60 cells affects the toxicity of compound 11 as well as that of reference compound 4. We observed that while the effect of compound 4 was not dependent on the type of the serum used, the toxicity of 11 was fully inhibited in the HS-containing medium even at the highest concentration of the drug used (50 µM). Furthermore, in experiments with CLL cells compound 11 exhibited no toxicity at concentrations of up to 10 µM (data not shown). These data indicate that HS is able to scavenge in some way compound 11. Since the only structural difference between compounds 4 and 11 is the presence of a strongly electrophilic CH₂Cl group in the latter case, we can suggest that the scavenging occurs due to alkylation of nucleophilic centers of HS by compound 11.
Independent of the mechanism of the latter process, it may preclude the possible anticancer activity of compound 11 \textit{in vivo}. Therefore, this prodrug was not selected for further biological tests.

It is worth noting that neither the substitution of 3- and 4-H-atoms in the phenyl residue for either electron donating or accepting moieties (compounds 5, 6, 7) nor the replacement of the phenyl rest with 2- and 3-pyridyl moieties (compounds 8, 9) affected the activity of aminoferrrocenes substantially. Furthermore, substitution of the N-benzyl residue for N-diphenylmethyl (compound 10) only slightly enhanced the activity towards the cancer cell lines RAJI (IC$_{50}$= 21 ± 4 µM for 10 \textit{versus} 35 ± 6 µM for 4, p<0.05) and BL-2 (IC$_{50}$= 16 ± 1 µM for 10 \textit{versus} 25 ± 1 µM for 4, p<0.01), whereas its effect on two other cell lines (HL-60 and JVM-2) was found to be negligible (Table 1). Unfortunately, the N-benzylation of the carbamate group of a 1,1'-bis-aminoferrrocene-derived prodrug did not exhibit the analogous favorable effect as the same derivatization of the mono-aminoferrrocenes (compare 13, 14 and 3, 4, Table 1).

We observed that the resistance of wt p53-expressing BL-2 and JVM-2 cell lines as well as mut p53-expressing RAJI cell line towards aminoferrrocenes is similar for the majority of the prodrugs studied here, except that in a few cases (compounds 7, 8 and 9) the BL-2 cell line was found to be slightly more sensitive than RAJI and JVM-2 cell lines (Table 1). Overall, these data support the hypothesis that the anticancer activity of aminoferrrocene-based prodrugs does not require p53 activity.

**Effect of aminoferrrocenes on the viability of wild type and TP53-mutated/deleted chronic lymphocytic leukemia cells**

Subsequently, we investigated the effects of two representative aminoferrrocene-based prodrugs 3 and 4 on the viability of CLL cells isolated from 25 patients. In this group 8 patients had p53-
mutated CLL cells and 17 patients had wild type p53 CLL cells. The effect of compounds 3 and 4, as well as of fludarabine on the viability of CLL cells was determined by using ATPlite™ Luminescence Assay System (PerkinElmer, Waltham, USA). Fludarabine is a purine analogue, which is currently used as a standard treatment of the CLL disease. The mode of action of this drug depends upon p53, making it more active against wt p53 cancer types. Therefore, treatment of CLL patients with fludarabine can cause a selective survival of p53-mutant CLL cells, which are more resistant and, therefore, less responsive to the therapy. We observed that after treatment of p53-mutated CLL cells (8 patients) with fludarabine (10 µM) for 48 h 48.7 ± 16.1 % viable cells remained in the suspension, whereas the same treatment of wild type p53 CLL reduced the number of viable cells to 34.7 ± 18.1 % (17 patients, Figure 2).

**Figure 2.** Mutated p53 CLL cells (mut TP53, 8 patients) or wild type CLL cells (wt TP53, 17 patients) were treated with prodrugs 3, 4 or fludarabine (each 10 µM) for 48 h and the % of viable cells in suspension was determined as described in the experimental section and plotted as the relative number of viable cells (the total number of control untreated cells was taken as 100 %).
The response of the cells of the selected, small group of patients to fludarabine was strongly heterogeneous, which is reflected in large standard deviations of the number of viable cells in populations of treated CLL cells – 16.1 % for p53-mutated CLL and 18.1 % for wild type p53 CLL. Therefore, the difference in activities of this drug towards mut p53 CLL and wt CLL cells observed is not significant within the observed experimental error (Student’s t-test).

Aminoferrocenes 3 and 4 exhibited in general a higher toxicity towards CLL cells than fludarabine (p<0.001): 9.1-12.5 % viable cells left after their treatment with these prodrugs (10 µM) for 48 h (compare with 39.2 % obtained after the treatment of CLL cells with fludarabine). Their toxicity turned out to be practically independent of the status of p53 in CLL cells (Figure 2). These data are in agreement with our results obtained for B-cell-derived cell lines (see above) and support the hypothesis that the mode of action of aminoferrocenes is not dependent upon p53. This advantageous property can make these prodrugs potentially suitable for the treatment of p53-mutated CLL and other hematological cancers.

**Ex vivo and in vivo toxicity of aminoferrocene 4**

Based on the preliminary studies obtained so far, we selected compound 4 for further evaluation of its toxicity *ex vivo* and *in vivo*. We have previously shown that this prodrug is not toxic towards normal (non-cancer) cells including fibroblasts (< 100 µM) and primary mononuclear cells (MNC’s, ≤ 10 µM). In order to validate these *in vitro* data in a more complex experimental model, we first investigated the toxicity of compound 4 *ex vivo* using rat precision-cut liver slices (PCLS) according to established procedures. PCLS are viable explants of tissue with a reproducible and well-defined thickness, containing cells in their natural environment. Notably, this technique is an FDA-approved model for drug toxicity and metabolism studies, and
it has been applied already for the assessment of toxicity of cisplatin\textsuperscript{21} and experimental gold-based compounds.\textsuperscript{22} In this assay liver cells remain in their natural environment and therefore, the obtained toxicity results are expected to be more relevant than those obtained earlier in experiments with cell cultures.

Thus, rat liver slices were cut and pre-incubated either for 1 h or 24 h before exposure to the prodrug as detailed in the experimental section, to allow the cells to recover from the cold ischemia and the slicing procedure. After pre-incubation, slices were incubated in fresh WEGG medium containing 2.5 µM, 5 µM or 10 µM of compound 4 for a period of 24 h. Afterwards they were collected and the viability of the tissues was determined measuring the ATP content.

The choice of the pre-incubation time appeared to be crucial: A marked decrease in the viability of 1 h pre-incubated slices was already found at a concentration of 5 µM prodrug (Figure 3). In contrast, slices pre-incubated for 24 h remained practically fully viable (83 %) even at the highest tested concentration (10 µM, Figure 3). Since aminoferrocenes are activated and as such expected to be more toxic under oxidative conditions\textsuperscript{9}, we may speculate that after 1 hour pre-incubation the slices are subject to oxidative stress due to the organ excision and slicing procedure increasing their sensitivity to compound 4. Indeed, others have reported that shortly after slice preparation the GSH concentration in the slices is low.\textsuperscript{23} In contrast, after 24 h pre-incubation slices reach a more physiological equilibrium, which is reflected in normal intracellular GSH concentration.\textsuperscript{23} Preliminary experiments exploring the toxicity of cisplatin in liver slices have shown that no effect of pre-incubation exists for this compound (data not shown). This indicates that the effect of pre-incubation is probably related to the specific toxic mechanism of aminoferrocenes. Taken together these data confirm that in normal (non-malignant) cells, which are characterized by low ROS concentration, aminoferrocenes remain
inactive. Furthermore, experiments were carried out with ferrocene 15 as a negative control and 17 as a positive control (Scheme 2). Both compounds were added to slices pre-incubated for 24 h, and incubation was continued for additional 24 h. As expected, ferrocene 15 showed no reduction in viability even at 40 µM, whereas compound 17 showed a high toxicity with an IC₅₀ of ca. 1 µM.

Based on the promising in vitro and ex vivo data on the toxicity of prodrug 4, we decided to further study its effects on healthy hybrid male mice BDF1 (DBA/2,♀ x C57Bl/6, ♂) at variable doses between 10 and 6000 µg/kg as described in the experimental section. Each dose was introduced via an intraperitoneal injection. One group contained 6 animals, which were monitored for 21 days after the drug injection. Every third day animals were weighted. All animals survived this treatment. Their weight increased by 1.8-2.5 g during the time of the experiment. The same weight increase was observed in the control group, which received no

![Figure 3](Figure.png) Viability of precision cut liver slices (PCLS) after 24 h of incubation with the aminoferrocene-based prodrug 4 and positive control 17 following 1 h (1 h pre) or 24 h of pre-incubation (24 h pre).
treatment. These data suggest that prodrug 4 exhibits no fulminant toxicity \textit{in vivo} at concentrations of up to 6 mg/kg, although more subtle effects cannot be excluded.

\textbf{Antitumor activity of prodrug 4 \textit{in vivo}}

Finally, antitumor activity of 4 was evaluated on hybrid male mice BDF1 (DBA/2,♀ x C57Bl/6, ♂), which carry L1210 leukemia, expressing mutant p53.\textsuperscript{17} In an exploratory experiment the treatment of mice (15 animals per group) was started on the second day after injection of L1210 cells. It included six daily intraperitoneal injections of compound 4 at a dose of 26 µg/kg in PBS solution (0.5 mL) containing 0.2 % (v/v) DMSO (total dose: 0.156 mg/kg). The control groups received the same volume of PBS solution. We observed that survival of the mice was extended from 13.7 ± 0.6 days in the control group to 17.5 ± 0.7 days in the treated group (p<0.05). This corresponds to 28 % lifetime extension that according to Sofina \textit{et al}\textsuperscript{24} indicates a significant antitumor effect thereby justifying further investigations of prodrug 4. It has been previously reported that 7 days after intraperitoneal injection of L1210 in BDF1 mice (DBA/2,♀ x C57Bl/6, ♂) ascites fluid of the animals contains over 93 % of L1210 cells with respect to the total cell number.\textsuperscript{25} In order to evaluate the drug-induced oxidative stress, we took probes of ascites fluid of treated and control groups and analyzed mitochondria integrity (JC-1 assay) and the intracellular ROS amount (CM-H2DCF DA assay) in the L1210 cells that were present in this sample. In particular, the fluorescence of L1210 cells isolated from the drug-treated animals and incubated with the JC-1 probe was found to be 97.0 ± 4.3 arbitrary units (a.u.), whereas the control probe (cells isolated from untreated animals) exhibited significantly (p<0.05) lower fluorescence of 48.2 ± 3.1 a.u. These data indicate the disturbance of the function of mitochondria in the cancer cells in the treated animals. Furthermore, we observed that L1210
cells from the treated animals, which were incubated with CM-H2DCF DA dye (230.0 ± 18.5 a.u.), were more fluorescent than those from the control group (163.3 ± 20 a.u., p< 0.05). These data indicate that the treatment with compound 4 enhances the oxidative stress in cancer cells in vivo.

CONCLUSIONS

We developed a substantially improved method for synthesis of N-substituted aminoferrocene-based prodrugs, which leads to yields of 72-91 %. In contrast, by using the previous method these compounds could be obtained with yields of under 5 %. In the key step, easily accessible starting material 4-(ferrocenylaminocarbonyloxymethyl)-phenylboronic acid pinacol ester was alkylated by variable benzyl chlorides or bromides at 22 ºC, whereas in the previously used protocol prolonged heating at 120 ºC was required. Therefore, heat sensitive building blocks could be introduced by the new approach, including for example an N-(4-acetoxyphenyl)methyl substituent. Overall, nine new N-substituted prodrugs were prepared by this protocol, which were obtained in high purity and amount and required limited purification. Thus, we were able to characterize the biological effects of the new series of compounds in vitro, ex vivo and in vivo. First, we observed that the prodrugs exhibit similar toxicity towards B-cell derived hematological cancer cell lines (JVM-2, RAJI and BL-2) having different p53 status. Subsequently, we confirmed that the toxicity of aminoferrocenes is practically independent from the p53 status in primary CLL cells. In preparation to the in vivo studies of the antitumor activity of the prodrugs, we confirmed that compound 4 exhibits practically no toxicity up to 10 µM in precision cut liver slices pre-incubated for 24 h up (ex vivo), and up to 6 mg/kg in hybrid male mice BDF1 (DBA/2,♀ x C57Bl/6, ♂) (in vivo). Finally, the antitumor activity of the most active
prodrug 4 was studied in hybrid male mice BDF1 (DBA/2,♀ x C57Bl/6, ♂), which carry L1210 leukemia. We observed that compound 4 administered in 6 daily doses of 26 µg/kg extends the survival of the mice from 13.7 ± 0.6 days to 17.5 ± 0.7 days. The prolonged survival was accompanied by increased oxidative stress of and membrane damage in L1210 cells isolated from the ascites fluid of treated animals. It is worth mentioning that the dose used in the in vivo antitumor experiment is much lower than the maximum texted dose of the in vivo toxicity studies, where no weight loss of the animals has been observed. Although further studies are necessary to validate the mechanisms of action of this new class of compounds, as well as more advanced toxicity studies, our findings indicate that these aminoferrocene prodrugs can potentially be applied for the treatment of cancers, which express mutated or no p53 and are often poorly responsive to the conventional chemotherapy.

EXPERIMENTAL SECTION

General Information

Commercially available chemicals of the best quality from Aldrich/Sigma/Fluka (Germany) were obtained and used without purification. Starting materials 1-3 (Scheme 1), 13 (Scheme 2) and control compounds 16 and 17 (Scheme 2) were prepared as described previously.⁹ NMR spectra were acquired on a Bruker Avance 400 or Bruker Avance III 600 spectrometer. ESI mass spectra were recorded on a Bruker ESI MicroTOF II. C, H, and N analysis was performed in the microanalytical laboratory of the chemical institute of the Fiedrich-Alexander-University of Erlangen-Nürnberg. UV-vis spectra were measured on a Lambda Bio+ UV/Vis spectrophotometer (Perkin Elmer) by using quartz glass cuvettes (Hellma GmbH, Germany) with a sample volume of 1 mL or micro-cuvettes with a sample volume of 100 µL (BRAND GmbH,
Germany). The fluorescence of live cells was quantified using a Guava easyCyte™ 6-2L Flow cytometer from Merck Millipore. The data were processed using the inCyte™ software package from Merck Millipore and the ModFIT LT™ software from Verity Software House. The purity of the prodrugs used in the biological tests was determined by C, H, and N analysis. According to these data, the purity of the prodrugs and controls was greater than 95%. Crystal structure data for prodrug 11 (Figure 1) were submitted to the Cambridge Structural Database (CSD). This structure was assigned the deposition number CCDC 1011896.

**Synthesis**

*Compound 4, protocol 1.* Compound 3 (0.5 g, 1.08 mmol) was dissolved in anhydrous DMF (20 mL) under nitrogen atmosphere. Then Cs₂CO₃ (1.06 g, 3.25 mmol) and tetra-butylammonium iodide (TBAI, 1.20 g, 3.25 mmol) were added to the solution. After stirring for 30 minutes at 22 °C, benzyl chloride (412 mg, 374 µL, 3.25 mmol) was added. The mixture was stirred for 18 h at 22 °C. Then, the solvent was removed in vacuum (0.01 mbar) and the product was purified by column chromatography on silica gel using petroleum ether/acetone (10/1, v/v) as eluent yielding an orange oil. Triturating of this material with hexane gave the product as an orange solid. Yield: 427 mg, 0.77 mmol (71%). TLC (SiO₂, eluent petroleum ether/acetone, 4:1, v/v) Rf = 0.5; ¹H-NMR (acetone-d₆, 300 MHz) δ (ppm) 7.73 (d, 2 H), 7.39-7.27 (m, 7 H), 5.25 (s, 2 H), 5.02 (s, 2 H), 4.46 (s, 2 H), 4.12 (s, 5 H), 3.97 (s, 2 H), 1.34 (s, 12 H). The spectra are in agreement with those reported for this compound.⁹

*Compound 4, protocol 2.* Compound 3 (50.0 mg, 108 µmol) was dissolved in anhydrous DMF (2 mL) under a nitrogen atmosphere. The solution was treated with NaH (5.20 mg, 130 µmol, 60 % mineral oil suspended) and stirred 30 min at 22 °C. Benzyl bromide (27.8 mg, 19.3 µL 163
μmol) was added and the reaction was stirred for 2 h at 22 °C. Then, the solvent was removed in vacuum (0.01 mbar) and the product was purified by column chromatography on silica gel using petroleum ether/acetone (4/1, v/v) as eluent. Yield: 55 mg, 100 μmol (92%).

Compound 5 was prepared analogously to compound 4 using protocol 1. The product was obtained as orange solid. Yield: 253 mg, 444 μmol (68%). TLC (SiO₂, eluent petroleum ether/acetone, 4/1, v/v) R_f = 0.5; ¹H-NMR (300 MHz, aceton-d₆): δ (ppm) 7.75 (d, 2H), 7.41 (m, 3H), 7.19-7.03 (m, 3H), 5.27 (s, 2H), 5.05 (s, 2H), 4.47 (s, 2H), 4.15 (s, 5H), 4.00 (s, 2H), 1.35 (s, 12H); ¹³C-NMR (101 MHz, aceton-d₆): δ (ppm) 164.65, 162.23, 142.57, 140.30, 135.11, 130.78, 127.47, 122.65, 114.02, 113.81, 113.65, 113.43, 84.08, 69.28, 67.49, 64.68, 62.81, 62.75, 24.71; HR-MS (ESI +), m/z: calculated for C₃₁H₃₃BFFeNO₄ [M-e⁻]+ 569.1836, found 569.1826; C, H, N analysis: calculated for C₃₁H₃₃BFFeNO₄ (%) – C 65.41, H 5.84, N 2.46; found – C 65.39, H 5.96, N 2.56.

Compound 6 was prepared analogously to compound 4 using protocol 1. The product was obtained as orange oil. Yield: 274 mg, 471 μmol (73%). TLC (SiO₂, eluent petroleum ether/acetone, 4/1, v/v) R_f = 0.51; ¹H-NMR (400 MHz, aceton-d₆): δ (ppm) 7.75 (d, 2H), 7.4-7.27 (m, 3H), 6.92-6.84 (m, 3H), 5.27 (s, 2H), 5.00 (s, 2H), 4.47 (s, 2H), 4.14 (s, 5H), 3.99 (s, 2H), 1.35 (s, 12H); ¹³C-NMR (101 MHz, aceton-d₆): δ (ppm) 160.49, 155.01, 141.03, 140.41, 135.14, 129.92, 127.50, 118.84, 112.55, 112.46, 101.79, 84.09, 69.28, 68.86, 67.37, 64.59, 62.82, 54.98, 53.71, 24.76; HR-MS (ESI+), m/z: calculated for C₃₂H₃₆BFNO₅ [M-e⁻]+ 518.2036, found 581.2030; C, H, N analysis: calculated for C₃₂H₃₆BFNO₅ (%) – C 66.12, H 6.24, N 2.41; found – C 65.69, H 6.25, N 2.43.
**Compound 7** was prepared analogously to compound 4 using protocol 1. The product was obtained as orange oil. Yield: 36 mg, 59 µmol (9%). TLC (SiO$_2$, eluent petroleum ether/acetone, 6/1, v/v) $R_f$ = 0.29; $^1$H-NMR (300 MHz, aceton-$d_6$): $\delta$ (ppm) 7.77 (d, 2 H), 7.37 (m, 4 H), 7.12 (d, 2 H), 5.27 (s, 2 H),  5.02 (s, 2 H),  4.48 (s, 2 H),  4.14 (s, 5 H),  3.99 (s, 2 H),  2.27 (s, 3 H),  1.35 (s, 5 H); $^{13}$C-NMR (75 MHz, aceton-$d_6$): $\delta$ (ppm) 168.30, 156.22, 14λ.51, 13λ.47, 135.85, 134.27, 126.99, 126.76, 121.47, 121.19, 83.20, 69.31, 68.71, 68.44, 68.35, 68.06, 63.73, 23.84, 19.62; HR-MS (ESI+), m/z: calculated for C$_{33}$H$_{36}$BFeNO$_6$ [M-e$^-$]$^+ 609.1986$, found 609.1984; C, H, N analysis: calculated for C$_{33}$H$_{36}$BFeNO$_6$ (%) – C 65.05, H 5.96, N 2.30; found – C 63.31, H 6.04, N 2.26.

**Compound 8** was prepared analogously to compound 4 using protocol 1. TLC (SiO$_2$, eluent petroleum ether/acetone, 4/1, v/v) $R_f$ = 0.36; $^1$H-NMR (300 MHz, aceton-$d_6$): $\delta$ (ppm) 8.57 (d, 2H), 7.78-7.70 (m, 3 H), 7.3-7.27 (m, 4 H), 5.22 (s, 2 H),  5.07 (s, 2 H),  4.48 (s, 2 H),  4.11 (s, 5 H),  3.98 (s, 2 H),  1.33 (s, 12 H); HR-MS (ESI+), m/z: calculated for C$_{30}$H$_{33}$BFeN$_2$O$_4$ 552.1883, found 552.1891.

**Compound 9** was prepared analogously to compound 4 using protocol 1. The product was obtained as orange oil. Yield: 84 mg, 152 µmol (23%). TLC (SiO$_2$, eluent petroleum ether/acetone, 4/1, v/v) $R_f$ = 0.35; $^1$H-NMR (300 MHz, aceton-$d_6$): $\delta$ (ppm) 8.60 (d, 2H), 8.48 (dd, 2H) 7.75-7.68 (m, 3 H), 7.38-7.32 (m, 3 H), 5.25 (s, 2 H),  5.05 (s, 2 H),  4.46 (s, 2 H),  4.12 (s, 5 H),  4.00 (s, 2 H),  1.33 (s, 12 H); $^{13}$C-NMR (75 MHz, aceton-$d_6$): $\delta$ (ppm) 155.28, 149.36, 149.26, 140.72, 135.61, 135.23, 134.85, 128.00 (two overlapping peaks), 124.20, 102.15, 84.57, 69.77, 68.03, 65.23, 63.25, 52.07, 25.20; HR-MS (ESI+), m/z: calculated for C$_{30}$H$_{33}$BFeN$_2$O$_4$ [M-e$^-$]$^+ 552.18827$, found 552.18783; C, H, N analysis: calculated for C$_{30}$H$_{33}$BFeN$_2$O$_4$ (%) – C 65.25, H 6.02, N 5.07; found – C 65.28, H 5.97, N 5.08.
**Compound 10.** Compound 3 (1.0 g, 2.17 mmol) was dissolved in anhydrous DMF (12 mL) under a nitrogen atmosphere. Then Cs$_2$CO$_3$ (4.24 g, 13.01 mmol) and TBAI (4.79, 13.01 mmol) were added to the solution. After stirring for 30 minutes at ambient temperature, diphenylmethyl chloride (2.31 mL, 2.64 g, 13.01 mmol) was added. The mixture was stirred for 7 days at 22 °C. Then, the solvent was removed in vacuum (0.01 mbar) and the product was purified by column chromatography on silica gel using DCM - DCM/ethylacetate (4/1, v/v) followed by a second column using petroleum ether/acetone (6/1, v/v). The product was isolated as orange solid. Yield: 78 mg, 124 µmol (6%). TLC (SiO$_2$, eluent petroleum ether/acetone, 4/1, v/v) R$_f$ = 0.5; $^1$H-NMR (400 MHz, aceton-d$_6$): $\delta$ (ppm) 7.70 (d, 2 H), 7.39- 7.30 (m, 2 H), 7.23 (d, 2 H), 6.76 (s, 1 H), 5.11 (s, 2 H), 4.33 (s, 2 H), 4.10 (s, 5 H), 3.99 (s, 2 H), 1.35 (s, 12 H); $^{13}$C-NMR (101 MHz, aceton-d$_6$): $\delta$ (ppm) 154.87, 140.64, 140.24, 135.03, 128.95, 128.46, 127.48, 127.44, 126.87, 102.65, 84.08, 69.46, 69.35, 67.22, 64.73, 64.56, 24.77; HR-MS (ESI+), m/z: calculated for C$_{37}$H$_{38}$BFeNO$_4$ [M-e$^-\cdot$]$^+$ 627.22448, found 627.22496; C, H, N analysis: calculated for C$_{37}$H$_{38}$BFeNO$_4$ (%) – C 70.84, H 6.11, N 2.23; found – C 70.41, H 6.04, N 2.07.

**Compound 11.** Compound 3 (500 mg, 1.08 mmol) was dissolved in anhydrous DMF (25 mL) under a nitrogen atmosphere. Then Cs$_2$CO$_3$ (4.24 g, 13.01 mmol) and TBAI (4.79, 13.01 mmol) were added to the solution. After stirring for 30 minutes at 22 °C, 1,2-bis(chloromethyl)benzene (569 mg, 3.25 mmol) was added. The mixture was stirred for 18 hours at 22 °C. Then, the solvent was removed in vacuum (0.01 mbar) and the product was purified by column chromatography on silica gel using petroleum ether/acetone (6/1, v/v) as eluent. Further purification of the crude orange solid was carried out by recrystallization from aceton. Yield: 222 mg, 370 µmol (34%). TLC (SiO$_2$, eluent petroleum ether/acetone, 6/1, v/v) R$_f$ = 0.34; $^1$H-NMR (400 MHz, aceton-d$_6$): $\delta$ (ppm) 7.72 (d, 2 H), 7.50 (d, 1 H), 7.35 (m, 4 H), 7.20 (d, 1 H), 5.28 (s,
2 H), 5.25 (s, 2 H), 4.91 (s, 2 H), 4.47 (s, 2 H), 4.19 (s, 5 H), 4.01 (s, 2 H), 1.35 (s, 12 H); $^{13}$C-NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 154.24, 138.70, 136.50, 134.80, 134.46, 134.18, 133.25, 129.99, 128.96, 126.68, 125.01, 83.41, 69.76, 68.76, 67.21, 63.94, 62.14, 50.51, 43.82, 24.43; HR-MS (ESI+), m/z: calculated for C$_{32}$H$_{35}$BClFeNO$_4$ [M-e$^-$]$^+$ 599.16979, found 599.17064: C, H, N analysis: calculated for C$_{32}$H$_{35}$BClFeNO$_4$ (%) – C 64.09, H 5.88, N 2.34; found – C 64.19, H 5.51, N 2.28.

**Compound 12.** Compound 3 (500 mg, 1.08 mmol) was dissolved in anhydrous DMF (5 mL) under a nitrogen atmosphere. Then Cs$_2$CO$_3$ (883 mg, 2.71 mmol) and TBAI (799 mg, 2.17 mmol) were added to the solution. After stirring for 30 minutes at 22 °C, 1,2-bis(chloromethyl)benzene (114 mg, 651 µmol) was added. The mixture was stirred for 18 hours at 22 °C. Then, the solvent was removed in vacuum (0.01 mbar) and the product was purified by column chromatography on silica gel using petroleum ether/acetone (6/1, v/v) as eluent. Yield: 198 mg, 193 µmol (30%). TLC (SiO$_2$, eluent petroleum ether/acetone, 4/1, v/v) $R_f$ = 0.32; $^1$H-NMR (400 MHz, aceton-$_d_6$): $\delta$ (ppm) 7.74 (d, 4 H), 7.29 (m, 8 H), 5.24 (s, 4 H), 5.10 (s, 4 H), 4.45 (s, 4 H), 4.16 (s, 10 H), 4.00 (s, 4 H), 1.35 (s, 24 H); $^{13}$C-NMR (101 MHz, CDCl$_3$): $\delta$ (ppm) 154.68, 140.35, 135.41, 135.16, 127.29, 127.18, 125.66, 102.05, 84.10, 69.33, 67.42, 64.68, 62.91, 51.38, 24.76; HR-MS (ESI+), m/z: calculated for C$_{56}$H$_{62}$B$_2$Fe$_2$N$_2$O$_8$ [M-e$^-$]$^+$ 1024.34051, found 1024.33976; C, H, N analysis: calculated for C$_{56}$H$_{62}$B$_2$Fe$_2$N$_2$O$_8$ (%) – C 65.66, H 6.10, N 2.73; found – C 65.78, H 6.42, N 2.70.

**Compound 14.** Compound 13$^9$ was dissolved in anhydrous DMF (20 mL) under a nitrogen atmosphere. Then Cs$_2$CO$_3$ (1.10 g, 3.40 mmol) and TBAI (1.25 g, 3.40 mmol) were added to the solution. After stirring for 30 minutes at 22 °C, benzyl chloride (429 mg, 391 µL, 3.40 mmol) was added. The mixture was stirred for 18 hours at 22 °C. Then, the solvent was removed in
vacuum (0.01 mbar) and the product was purified by column chromatography on silica gel using petroleum ether/acetone (4/1, v/v) as eluent. Yield: 345 mg, 376 μmol (55%). TLC (SiO₂, eluent petroleum ether/acetone, 4/1, v/v) Rₓ = 0.46; ¹H-NMR (300 MHz, acetone-d₆): δ (ppm) 7.74 (d, 4 H), 7.24-7.35 (m, 14 H), 5.27 (s, 4 H), 4.98 (s, 4 H), 4.50 (s, 4 H), 3.95 (s, 4 H), 1.35 (s, 24 H); ¹³C-NMR (101 MHz, acetone-d₆): δ (ppm) = 140.35, 139.16, 135.16, 128.82, 128.46, 127.53, 127.24, 126.87, 126.70, 102.22, 84.08, 67.40, 65.72, 63.49, 53.04, 24.75; HR-MS (ESI+), m/z: calculated for C₅₂H₅₈B₂FeN₂O₈ [M - e]⁺ 916.3740, found 916.3743; C, H, N analysis: calculated for C₅₂H₅₈B₂FeN₂O₈ (%): C 68.15, H 6.38, N 3.06; found – C 68.09, H 6.45, N 3.11.

**Cellular Assays**

*Cells and Cell Culture.* The human promyelocytic leukemia cell line HL-60 was obtained from Sigma-Aldrich (Germany). Burkitt lymphoma cell lines RAJI, BL2 and human mantle cell lymphoma cell line JVM-2 were obtained from DSZM (Germany). Cells were cultured according to recommendations of DSMZ. In particular, RAJI and JVM-2 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. BL-2 cells were grown in RPMI 1640 medium supplemented with 20% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. All cells studied here are non-adherent. Stock suspensions of the cells were grown up to 0.5-1.5 x 10⁶ cells/mL and diluted as required.

*Estimation of oxidative stress in cell cultures.* An aliquot of cells was taken from the cultivation medium. The medium was replaced with PBS buffer to obtain a cell suspension containing 10⁶ cells/mL. A solution of 2’,7’-dichlorodihydrofluorescin diacetate (DCFH-DA, 1 μL, 5 mM in DMSO) was added to the cell suspension (1 mL) and incubated in the dark for 30 min at room temperature. Afterwards, the fluorescence of the samples (λₛₑₓ = 488 nm, λₛₑₘ = 530 nm) was determined by using the flow cytometer.
**Assay for Determination of Cell Permeability of the Prodrugs**

Cells grown in RPMI 1640 medium supplemented with 10% FBS (20% for BL-2), 1% glutamine, and 1% penicillin/streptomycin were centrifuged, and the medium was replaced with RPMI 1640 medium (5% FCS, 1% L-glutamine, 1% penicillin/streptomycin) to obtain suspensions containing 10^6 cells/mL. Solutions of prodrugs (10 μL, solvent DMSO) were added to the suspensions (1 mL) and incubated for 1 h. The final concentration of the prodrugs in the suspensions was 50 μM. Afterwards, cells were washed three times with PBS buffer (3 × 500 μL) and treated with concentrated H₂O₂ solution (200 μL, 1 M) for 30 min, and all volatiles were removed by lyophilization. Dry, lysed cells were washed with water (200 μL), and aqueous solution obtained was acidified with HCl (400 μL, 0.1 M). Then this solution was extracted with 2-ethyl-1,3-hexanediol (100 μL, 10% in CHCl₃, v/v), and a portion of the organic phase obtained (70 μL) was mixed with H₂SO₄/CH₃CO₂H (400 μL, 1/1, v/v). Curcumin solution in methyl isobutyl ketone (250 μL, 2 mg in 1 mL of the solvent) was added and allowed to react for 2 h. The reaction was quenched by addition of water (1 mL). The light absorbance at 550 and 780 nm of the organic phase was measured. The former value corresponds to absorbance of curcumin-boron complex, whereas the second one is taken as a baseline. The baseline corrected absorbance at 550 nm (A(550 nm) – A(780 nm)) was proportional to the concentration of boron in the mixture.

**Effect of the compounds on the viability of cell lines**

The cells were centrifuged, the medium was removed, and the cells were washed two times with PBS buffer and re-suspended in the RPMI 1640 medium containing 5% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. This suspension was spread in the wells of a 96-well microtiter plate (50 000 cells per well per 100 μL). Stock solutions of prodrugs of different concentrations
(1 µL, solvent DMSO, final concentrations in wells were 1, 5, 10, 20, 50 µM) were added to the wells and incubated for 48 h at 37 °C under 5% CO₂. Four experiments were conducted for each concentration of the prodrug. Finally, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 20 µL of the solution prepared by dissolving MTT (5 mg) in PBS buffer (1 mL)) was added to each well, incubated for 3 h, treated with sodium dodecyl sulfate (SDS) solution (90 µL, 10% solution in 0.01 M aqueous HCl), and incubated overnight. Afterwards, the intensity of the absorbance at 590 nm was measured. MTT is converted in live cells to blue dye with the absorbance maximum $\lambda_{\text{max}}$ at 590 nm. The absorbance at 690 nm was taken as a baseline value. The baseline corrected absorbance at 590 nm ($A(590 \text{ nm})-A(690 \text{ nm})$) was applied to calculate the relative number of viable cells. IC₅₀ values were determined by fitting the experimental data expressing the number of viable cells (%) OY-axis versus drug concentration (OX-axis) with a sigmoidal curve using a curve fitting system for Windows: CurveExpert 1.4.

Experiments with primary CLL cells were conducted as described elsewhere.⁹ The viability of CLL cells was determined by ATPlite™ Luminescence Assay System (PerkinElmer, Waltham, USA). The cells were incubated in medium containing human serum (HS). TP53 mutation status in CLL cells was determined as described elsewhere.²⁶,²⁷

Preparation of rat Precision-Cut Liver Slices (PCLS) and toxicity studies ex vivo. Male Wistar rats (Charles River, Sulzfeld, Germany) of 250-450 g were housed under a 12 h dark/light cycle at constant humidity and temperature. Animals were permitted free access to tap water and standard lab chow. All experiments were approved by the committee for care and use of laboratory animals of the University of Groningen and were performed according to strict governmental and international guidelines.
PCLS were made as described by de Graaf et al.\textsuperscript{20} Cores of tissue (5 mm) were prepared from the liver using a coring tool. The slices were prepared with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O\textsubscript{2} and 5% CO\textsubscript{2}). Liver slices (250 µm thick and 4 mg wet weight) were stored in ice-cold Krebs-Henseleit buffer until incubation. PCLS were incubated in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) at 37°C individually in 1.3 ml Williams’ medium E (WME, Gibco by Life Technologies, Paisley, UK) with glutamax-1, supplemented with 25 mM D-glucose (Gibco) and antibiotics (50 µg/ml gentamicin, Gibco) at pH 7.4 with shaking (90 times/min) in the atmosphere of carbogen. After 1 h or 24 h of pre-incubation the medium was replaced by medium containing the compounds under study. Stock solutions of compounds 4, 15 and 17 were prepared in dimethyl sulfoxide at a concentration of 10 mM (DMSO, VWR) and stored at 4°C. The final concentration of DMSO during the PCLS incubation was always below 1% to exclude DMSO toxicity. Concentration dependent toxicities of compounds were evaluated by incubating the rat PCLS for 24 h with different concentrations of compounds between 0 and 10 µM. Afterward, the slices were collected for ATP and protein determination, by snap freezing them in 1 ml of 70% ethanol/2 mM EDTA. The viability of PCLS was determined by measuring the ATP content using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) as described previously. The ATP content was corrected by the protein amount of each slice and expressed as pmol/µg protein. The protein content of the PCLS was determined by the Bio-Rad DC Protein Assay (Munich, Germany), using bovine serum albumin (BSA, Sigma-Aldrich) for the calibration curve. The ATP data were expressed as the relative value to the 24 h control tissue. Obtained values are mean ± SE of at least three independent experiments.
**In vivo experiments.** All experiments were conducted on male mice BDF1 (DBA/2,♀ x C57Bl/6, ♂) weighing 20-22 g, which were obtained from the vivarium of R. E. Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology of the National Academy of Sciences of Ukraine, in agreement with recommendations of the Committee on Bioethics at the Presidium of National Academy of Sciences of Ukraine. The prodrug 4 was dissolved in DMSO at 37 °C to obtain a 1 mM solution. For obtaining solutions of the required concentration this stock solution was diluted with PBS. For determination of the acute toxicity the diluted solutions were introduced once via intraperitoneal injections (solution volume 0.5 mL) at doses 10, 50, 100, 150, 200, 300, 400, 500, 700, 1000, 1500, 3000 and 6000 µg/kg. Each group contained 6 animals and was monitored for 3 weeks. Every third day animals were weighed. The weight of animals increased by 1.8-2.5 g in both control and treated groups during the time of the experiment. The control group received 0.5 mL of the physiological solution (PBS).

For determination of the antitumor activity the prodrug was introduced intraperitoneally everyday (for 6 days) starting from the second day after injection of L1210 cells. The latter cells (10⁶ cells/animal) were introduced intraperitoneally. The animals were divided in two groups, each containing 15 animals. The first group served as control and received 0.5 mL of the physiological solution (PBS). Animals in the other group were treated during 6 days with prodrug 4 at a dose of 26 µg/kg per day. 24 h after the last injection of the prodrug solution ascites fluid was taken out of animals. In this experimental model ascites fluid contained over 93 % of L1210 cells with respect to the total cell number.²⁵ The integrity of mitochondria (JC-1 assay, Life Technologies) and the concentration of ROS (CM-H2DCF DA assay, Life Technologies) were determined by using flow cytometry (Beckman Coulter, USA, excitation laser 488 nm, emission channels: FL1 – 525 nm, FL2 – 575 nm).
Measurements of intracellular ROS in L1210 cells: \(2.5 \times 10^5\) L1210 cells from ascites fluid were centrifugated at 1500 rpm for 5 minutes, re-suspended in PBS and incubated for 30 minutes at 37 °C with CM-H2DCFDA (10 mM). The mean fluorescence of the cells was determined using FL1 emission channel of the flow cytometer. The acquisition was performed on 10,000 gated events.

Analysis of mitochondrial transmembrane potential in L1210 cells: JC-1 forms J-aggregates in mitochondria of viable cells, which can be detected using FL2 channel. In apoptotic cells green monomeric JC-1 remains in the cytosol and can be detected using a FL1 channel. We used the ratio of J-aggregates to monomers (FL2 to FL1) as an indicator of the cellular mitochondrial transmembrane potential. In particular, L1210 cells \((2.5*10^5 / \text{mL})\) were stained with JC-1 (7.5 mM in PBS) for 10 minutes at 37° C. The mean fluorescence of the cells (FL2 and FL1) was then determined using a flow cytometer on the basis of the quadrant plot to distinguish monomers from J-aggregates. To set the quadrants, in a control experiment the cells were treated with H\(_2\)O\(_2\) (20 mM, 37 °C, 30 minutes) to obtain cells with depolarized mitochondrial membrane potential.

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ABBREVIATIONS

ADDP, 1,1’-(azidodicarbonyl)dipiperidine; Bipy, 2,2-bipyridine; BnCl, benzyl chloride; BSO, buthionine sulfoximine; CH₂Cl₂, dichloromethane; CLL, chronic lymphocytic leukemia; CM-H₂DCF-DA, 5(6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate; DCDFH, 2′,7′-
dichlorodihydrofluorescein; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; EBV, Epstein-Barr-Virus; EDTA, N,N,N′,N′-ethylenediaminetetraacetic acid; Fe, ferrocene; FCS, fetal calf serum; GSH, reduced glutathione; GSSG, oxidized glutathione; HL-60, human promyelocytic leukemia cells; 8-HQ, 8-hydroxyquinoline; HS, human serum; MCL, human mantle cell lymphoma; MNC, mononuclear cells; MOPS, 3-(N-morpholino)propanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NOASA, nitric oxide-donating aspirin; PCLS, rat precision cut liver slice; QM, quinone methide; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; TBAI, tert-butylamonium iodide; TLC, thin layer chromatography; wt, wild type; mut, mutated.

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\textbf{N-benzyl-substituted aminoferrocene-based prodrugs}

- p53-independent anticancer activity in cell lines and primary CLL cells
- non-toxic \textit{ex vivo} (up to 10 \textmu M, rat liver slices)
- non-toxic \textit{in vivo} (up to 6 mg/kg, BDF1 mice)
- anticancer activity \textit{in vivo} (L1210 leukemia in BDF1 mice)