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Aminoferrocene-Based Prodrugs and Their Effects on Human Normal and Cancer Cells as Well as Bacterial Cells

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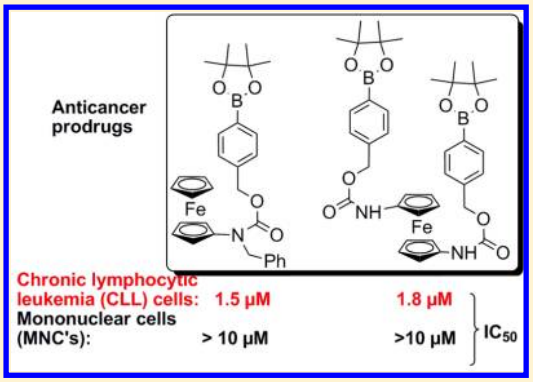
Supporting Information

ABSTRACT: Aminoferrocene-based prodrugs are activated under cancer-specific conditions (high concentration of reactive oxygen species, ROS) with the formation of glutathione scavengers (*p*-quinone methide) and ROS-generating iron complexes. Herein, we explored three structural modifications of these prodrugs in an attempt to improve their properties: (a) the attachment of a −COOH function to the ferrocene fragment leads to the improvement of water solubility and reactivity in vitro but also decreases cell-membrane permeability and biological activity, (b) the alkylation of the *N*-benzyl residue does not show any significant affect, and (c) the attachment of the second arylboronic acid fragment improves the toxicity (IC_{50}) of the prodrugs toward human promyelocytic leukemia cells (HL-60) from 52 to 12 μM. Finally, we demonstrated that the prodrugs are active against primary chronic lymphocytic leukemia (CLL) cells, with the best compounds exhibiting an IC_{50} value of 1.5 μM. The most active compounds were found to not affect mononuclear cells and representative bacterial cells.



INTRODUCTION

Cancer cells are formed as a result of genetic transformation of normal cells. Because of these alterations, an abnormal, cancer-specific microenvironment is created both in the cancer cells themselves and in the tumor tissues. These differences can be exploited to design cancer-specific drugs.[1] For example, the increased ROS level (ROS: $^1O_2$, O_2^-, H_2O_2, and HO•) is an especially attractive target for anticancer therapy because it seems to be a general feature of cancer.[2] Drugs targeting this feature can potentially exhibit activity against all cancer types. Several anticancer drugs have been described that act by increasing the ROS concentration in cancer cells beyond the apoptotic level and thereby inducing their death. These include arsenic trioxide,[3] fenretinide,[4] nitric oxide-donating aspirin (NO-ASA),[5] buthionine sulfoximine (BSO),[6] imexone,[7] motexafin gadolinium,[8] menadione,[9] β-lapachone,[10] deoxynyboquinone,[11] and others.[12] Although such compounds kill cancer cells efficiently, they also increase the ROS concentration in normal cells, thereby making the process of mutating native genomic DNA more probable. This side effect is dangerous because it can cause the induction of secondary malignancies. Prodrugs, which are activated only under cancer-specific conditions, should lack this disadvantage. Few anticancer prodrugs have been described up to date. For example, glycopeptides bleomycins are used as cytostatic agents in the chemotherapy of a variety of cancers.[13] They bind iron ions, which are available in cancer cells and to a lesser extent in normal cells, resulting in the formation of iron/bleomycin complexes. The latter compounds bind genomic DNA and induce its cleavage. Ferrocene derivative hydroxyferrocifen and its analogues, developed by the group of G. Jaouen, are activated in cells by oxidation with the formation of alkylating agents, which scavenge glutathione and thereby inhibit the cellular antioxidative system.[14] Furthermore, the group of C. Jacob has prepared organochalcogen-based compounds that are activated in the presence of elevated (cancer-specific) ROS with the formation of ROS-generating catalysts. The latter species induce strong oxidative stress in cells leading to their death.[15]

Cleavage of aryl- and alkylboronic acids and their esters in the presence of hydrogen peroxide has been used for a long time in synthetic organic chemistry in the last step of a

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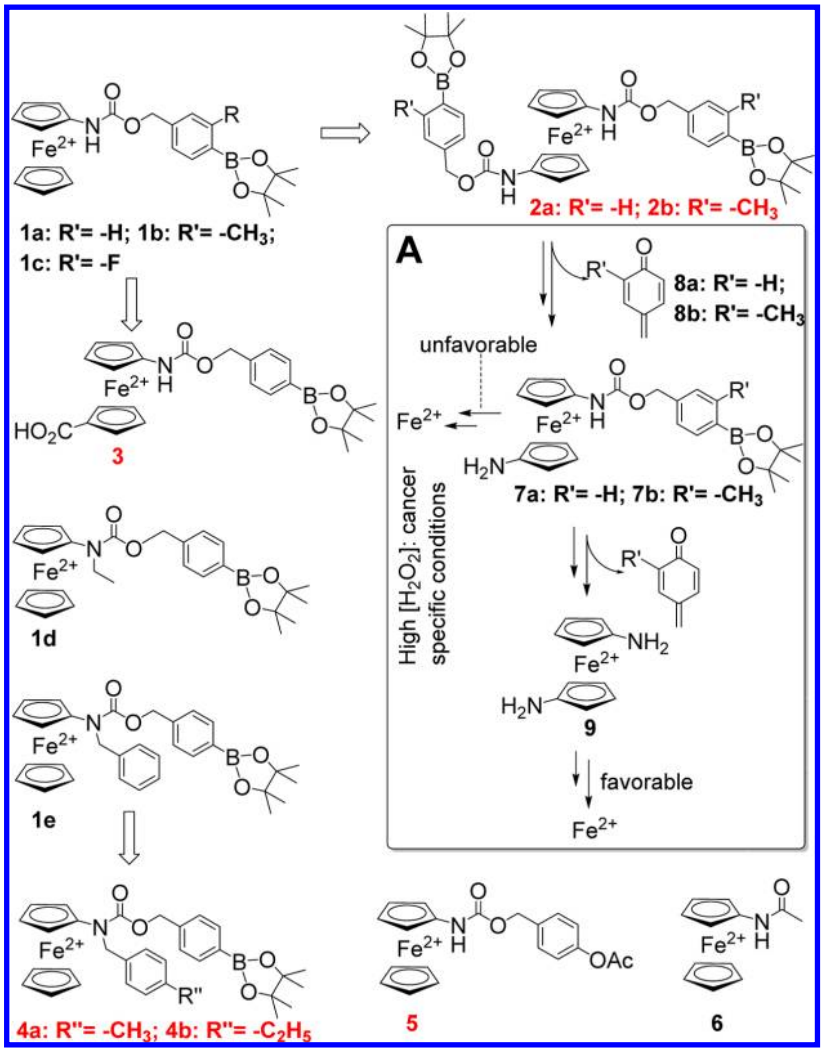
Scheme 1. Structures of Reported Aminoferrocene-Based Prodrugs 1a−e,[19] Their Analogues 2a, 2b, 3, 4a, and 4b, and a Positive Control, 5, Which Are Described in This Article[a]



[a]The suggested activation mechanism of prodrugs **2a** and **2b** is given in inset A. Compound **6** is a negative control described earlier. Compounds that are reported for the first time are indicated with red numbers.

hydroboration-oxidation reaction. Chang and co-workers have utilized such reactivity of boronic acid esters to design intracellular, fluorogenic hydrogen peroxide sensors.[16] The group of R. C. Hartley later applied this approach to prepare "caged" 2,4-dinitrophenol (DNP) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) derivatives. In particular, in the presence of hydrogen peroxide these compounds form DNP and FCCP, which exhibit biological activity as mitochondrial uncouplers (proton translocators).[17] Furthermore, the group of Peng has reported on hydrogen peroxide-inducible DNA cross-linking agents containing a di-(3-chloroethyl)-aminobenzyl fragment and an H_2O_2-reactive arylboronic acid pinacol ester.[18] Finally, we have described aminoferrocene-based prodrugs that are activated in leukemia cells because of the chemical reaction with reactive oxygen species like O_2^-, H_2O_2, and HO•.[19] In particular, the C−B bond of the boronic acid ester residue of the prodrugs is cleaved in the presence of ROS, resulting in the formation of two cytotoxic products: a quinone methide that inhibits the cellular antioxidative system and redox-active iron-containing species (aminoferrocenes and iron(II) ions) that are able to induce the catalytic generation of highly active ROS like O_2^- and HO• from molecular oxygen and hydrogen peroxide. The dual, synergistic effect of the reaction products (inhibition of the antioxidative system and catalytic generation of ROS) induces strong oxidative stress in the cells thereby causing their death. In contrast, in normal cells lacking a high ROS concentration, the aminoferrocene-based prodrugs remain inactive. Cancer-specific prodrugs activated by other mechanisms have been reviewed.[20]

Herein, we report the synthesis of five new derivatives of aminoferrocene-based prodrugs: **2a**, **2b**, **3**, **4a**, and **4b** as well as a positive control, **5**, that is activated cell-nonspecifically because of the reaction with intracellular esterases (Scheme 1). Our goal was to find modifications of lead structures **1a** and **1e** that lead to prodrugs with improved properties including cell membrane permeability sufficient for in vivo applications, solubility in water, high toxicity to cancer cells, and cancer-cell specificity. Furthermore, the toxicity of all prepared aminoferrocene-based prodrugs toward human promyelocytic leukemia (HL-60) cells, selected primary cancer cells (chronic lymphocytic leukemia, CLL), and related normal cells

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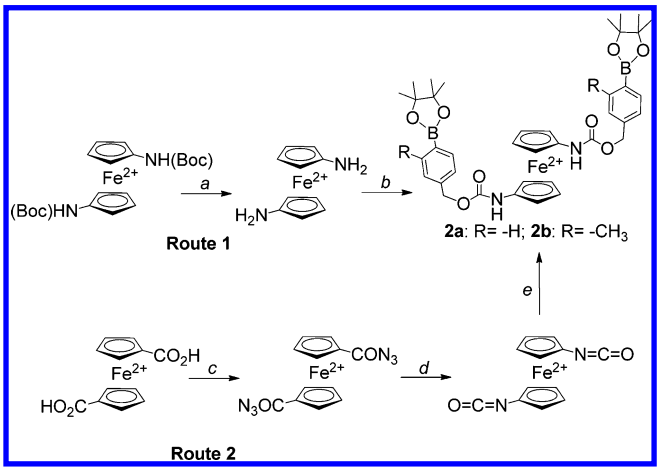
(mononuclear cells, MNC) as well as *Escherichia coli* (*E. coli*) and *Streptococcus agalactiae* (*S. agalactiae*) bacteria was evaluated. These studies were conducted to estimate the cancer-cell specificity of the prodrugs and to evaluate their potential for in vivo application.

RESULTS AND DISCUSSION

1,1′-Bis-aminoferrocene-Based Prodrugs. We assumed that the biological effect of known aminoferrocene-based prodrugs[19] could be further potentiated by attachment of the second arylboronic acid ester residue to the ferrocene core. Such compounds were expected to be activated by H_2O_2 more efficiently than the original prodrugs because of the presence of twice as many reactive groups. Moreover, twice as many *p*-quinone methide fragments could be potentially released from these prodrugs than from the earlier reported amino-ferrocenes,[19] which should lead to a stronger inhibition of the intracellular antioxidant system.

To test this hypothesis, we prepared two representative doubly substituted ferrocenes **2a** and **2b** (Schemes 1 and 2). Compound **2a** is an analogue of **1a**, whereas **2b** is an analogue of **1b**.

Scheme 2. Two Tested Routes (1 and 2) for the Synthesis of Bis-1,1′-aminoferrocene-Based Prodrugs 2a and 2b[a]



[a](a) CF_3CO_2H, 0 °C; (b) triphosgene, for the synthesis of **2a**: 4-(hydroxymethyl)phenyl-boronic acid pinacol ester, for the synthesis of **2b**: 4-hydroxymethyl)-2-methylphenyl-boronic acid pinacol ester, 90 °C (c) route 1, $C_2O_2Cl_2$; route 2, NaN_3, $(n\text{-}Bu)_4NBr$; (d) 100 °C, toluene; and (e) for the synthesis of **2a**: 4-(hydroxymethyl)phenyl-boronic acid pinacol ester, for the synthesis of **2b**: 4-(hydroxymethyl)-2-methylphenyl-boronic acid pinacol ester.

Prodrug **2b** is derived from **2a** by the formal substitution of two protons at the R′ position (Scheme 1) for two methyl groups. This compound was expected to be more lipophilic than **2a** and have a correspondingly better membrane permeability and increased toxicity. An analogous effect was observed earlier for related monomodified aminoferrocenes **1a** and **1b**.[19]

Synthesis. We first attempted to prepare bis-substituted compounds analogously to their monofunctionalized analogues by replacing aminoferrocene for 1,1′-diaminoferrocene (route 1, Scheme 2).[19] Because the latter compound is rather unstable, it was prepared immediately before the next reaction step by acidic deprotection of 1,1′-di(*tert*-butoxycarbonylamino)-ferrocene. In the following step, 1,1′-diaminoferrocene was reacted at an elevated temperature (120 °C) with triphosgene, the reaction mixture was cooled to 22 °C, and either 4-(hydroxymethyl)phenyl boronic acid pinacol ester (synthesis of **2a**) or 4-(hydroxymethyl)-2-methylphenyl-boronic acid pinacol ester (synthesis of **2b**) was added and left reacting for over a period of 26 h. Although all operations were conducted under anaerobic conditions, a large amount of a black-colored unidentifiable mixture of side products and only a minor amount of the desired bis-substituted aminoferrocenes were formed. After extensive column chromatography, less than 2% of the desired products could be isolated with ~80% purity according to ^{1}H NMR analysis. Control experiments with 1,1′-diaminoferrocene confirmed that this starting material was not stable under the reaction conditions employed. Attempts to improve the yield by optimizing the initial reaction temperature and using alternatives to triphosgene reagents, including 1,1′-carbonyldimidazole and 4-nitrophenyl chloroformate in combination with bases (NEt_3, DIEA, DMAP, and DBU) or without any bases, were not successful. Therefore, the alternative protocol for synthesis of **2a** and **2b** was developed in which the usage of unstable 1,1′-diaminoferrocene was avoided (route 2, Scheme 2). In particular, in the first step, known 1,1′-dicarboxyferrocene[21] was converted to 1,1′-diisocyanatoferrocene.[22] The following reaction with either 4-(hydroxymethyl)-phenyl boronic acid pinacol ester or 4-(hydroxymethyl)-2-methylphenyl boronic acid pinacol ester in CH_2Cl_2 furnished correspondingly prodrugs **2a** or **2b** (Scheme 2). The isolated yield of **2a** was 9% and that of **2b** was 18%. Both compounds were obtained with >95% purity after a single chromatographic purification.

Reaction of Bis-aminoferrocene-Based Prodrugs with Hydrogen Peroxide In Vitro and Their Effect on Human Promyelocytic Leukemia Cells (HL-60). It was sensible to assume that **2a** and **2b** would react with H_2O_2 analogously to that of the previously described monomodified aminoferrocene **1a** (Scheme 1). In particular, we expected that the activation of **2a** (or **2b**) would be triggered by the cleavage of one or two B–C bonds followed by the release of corresponding 1 or 2 equiv quinone methide **8a** (**8b**) as well as iron complexes **7a** (**7b**) and 1,1′-diaminoferrocene (**9**). The latter labile iron complexes could be spontaneously converted to iron ions. For the detection of these iron ions, we used a 2,2′-bipyridine assay described earlier (Table 1).[19] In short, a prodrug (0.1 mM) is dissolved in aqueous buffer (pH 7.5) and treated with H_2O_2 (1 mM) for 100 min. According to the chromatographic data, prodrugs **1a**, **1e**, **2a**, and **2b** are fully transformed into products under these experimental conditions. After the treatment with H_2O_2, sodium dithionite is added to convert Fe^{3+} ions into Fe^{2+} ions. Finally, 2,2-bipyridine (bipy) is added to bind the metal ions, resulting in the formation of a red-colored $[Fe(bipy)_3]^{2+}$ complex. The amount of this compound is quantified using UV–vis spectroscopy. We observed that the yield of iron ions released from prodrugs **2a** and **2b** does not exceed 20%, which indicates the formation of other iron-containing species as the major products (entries 2 and 3, Table 1). Under the same conditions, parent prodrug **1a** (entry 1) liberates only iron ions, whereas *N*-benzyl substituted prodrug **1e** releases only 9% of iron ions (entry 5, Table 1). In our previous publication, we provided spectroscopic evidence that over 90% of **1e** is transformed into stable *N*-benzylaminoferrocene under the oxidative conditions. The possible stable products resulting from **2a** and **2b** could be intermediates **7a**, **7b**, and 1,1′-

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Table 1. Efficiency of Activation of Aminoferrocene Prodrugs and Control Compounds In Vitro

entry	drug	efficacy of Fe release (% of $FeSO_4$)[a]	efficacy of ROS release (% of $FeSO_4$)[b]
1	**1a**	95 ± 2	87 ± 1
2	**2a**	19 ± 7	66 ± 5
3	**2b**	10 ± 6	23 ± 3
4	**3**	100 ± 5	106 ± 7
5	**1e**	9 ± 4	53 ± 4
6	**4a**	15 ± 4	22 ± 19
7	**4b**	12 ± 4	23 ± 3
8	**6**	1 ± 1	10 ± 5
9	$FeSO_4$	100	100

[a]The amount of iron ions released from prodrugs (0.1 mM) in the presence of H_2O_2 (1 mM) in aqueous *N*-morpholinopropane-sulfonic acid buffer (MOPS, 100 mM, pH 7.5) for 100 min was determined using a 2,2-bipyridine-based assay.[19] The amount of iron released from $FeSO_4$ (0.1 mM) was used as a reference (100%). The standard deviations of these values are <7% [b]ROS release efficacy was determined using the following equation: 100% × (F − F_0)/(F($FeSO_4$) − F_0), where F is the fluorescence of the mixture of H_2O_2 (10 mM), glutathione (GSH, 5 mM), ethylenediamine tetracetic acid (EDTA, 10 mM), 2′,7′-dichlorodihydrofluorescein (DCDFH, 0.1 mM), and prodrug (0.1 mM), F($FeSO_4$) is the fluorescence of the same mixture where the prodrug was substituted for $FeSO_4$ (0.1 mM), and F_0 is the fluorescence of the mixture lacking any iron-containing substance. The fluorescence intensity (λ_{ex}= 501 nm and λ_{em}= 531 nm) was measured 40 min after the addition of the iron source to the solution of other components. The standard deviations of these values are <9%

diaminoferrocene (Scheme 1). Because the latter compound was available in our laboratory, we conducted an exploratory experiment in which it was subjected to the conditions of the 2,2′-bipyridine assay for 100 min. We observed that 1,1′-aminoferrocene was completely decomposed with the formation of iron ions. These data indicate that 1,1′-aminoferrocene cannot be formed as a major product as a result of the H_2O_2-induced activation of **2a** and **2b**. Therefore, it was sensible to assume that >80% of **2a** and **2b** are converted into the cleavage products of one 4-methylphenylboronic acid pinacol ester residue, **7a**, and **7b**, respectively. The formation of compound **7a** in solutions of prodrug **2a** (1 μM) containing H_2O_2 (0.1−1 mM) was experimentally confirmed by ESI-mass spectrometry (data not shown). We speculate that **7a** and **7b** are more stable than 1,1′-diaminoferrocene and aminoferrocene because of the electron-acceptor effect of a carbamate residue.

Iron-containing species released from the prodrugs in the presence of H_2O_2, which include aminoferrocenes **7a** or **7b** (>80%) and iron ions (10−19%, entries 2 and 3, Table 1), are capable of catalyzing the generation of highly reactive oxygen species like HO• and O_2^- from less reactive H_2O_2 and molecular oxygen. These products are especially toxic and can cause cell death. We observed earlier that the efficacy of the generation of such ROS in cells correlates with cytotoxicity of aminoferrocene-based prodrugs.[19] Therefore, the ability of new prodrugs **2a** and **2b** to induce the formation of the ROS was evaluated. In this experiment, we used nonfluorescent 2′,7′-dichlorodihydrofluorescein (DCDFH), which is converted into a fluorescent product in the presence of HO• and O_2^- that allows for the detection of the latter species using fluorescence spectroscopy. In a typical experiment,[19] a solution of H_2O_2 (10 mM), glutathione (GSH, 5 mM), ethylenediamine tetracetic acid (EDTA, 10 mM), and DCDFH (0.1 mM) was prepared. Next, a prodrug or one of the control compounds (0.1 mM) was added, and after 37 min the increase in the fluorescence intensity at 531 nm (λ_{ex} = 501 nm) was determined. Representative kinetic data for a selected prodrug and the control compounds are shown in Figure 1. We defined the

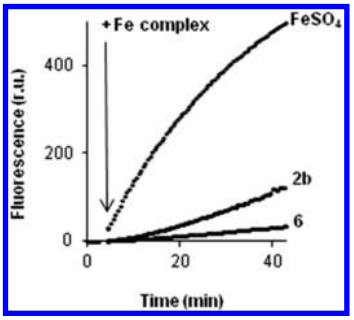

Figure 1. Monitoring of the generation of ROS in the presence of a representative prodrug, **2b**, a negative control, **6**, and a positive control, $FeSO_4$, using fluorescence spectroscopy in combination with a ROS-sensitive fluorogenic reagent, 2′,7′-dichlorodihydrofluorescein (DCDFH). The fluorescence (λ_{ex}= 501 nm and λ_{em}= 531 nm) of a mixture of H_2O_2 (10 mM), glutathione (GSH, 5 mM), ethylenediamine tetracetic acid (EDTA, 10 mM), and DCDFH (0.1 mM) was monitored for the first 2 min. The Fe-containing complex (0.1 mM) was then added (the addition time is indicated with an arrow) and the fluorescence was monitored for a further 40 min.

efficacy of ROS generation by a particular prodrug as a ratio 100% × (F(prodrug) − F_0)/(F($FeSO_4$) − F_0), where F(prodrug) and F($FeSO_4$) are the fluorescence intensities obtained in the presence of a prodrug and $FeSO_4$ (0.1 mM), respectively, and F_0 is the fluorescence of the mixture lacking any iron-containing substance. These values correspond to the percent activity with respect to the positive control, $FeSO_4$ (Table 1). We observed that compounds **2a** and **2b** exhibited 66 ± 5 and 23 ± 3% of the activity of $FeSO_4$, respectively, which is comparable to that of **1e** (24%) and **1a** (56%, Table 1). According to these data, doubly modified ferrocenes **2a** and **2b** could potentially exhibit anticancer activity analogously to that of previously studied **1a** and **1e**. To evaluate whether this is indeed a case, we studied the cell toxicity of the new prodrugs toward human promyelocytic leukemia cells (HL-60). This cell line was selected because data on the effects of the parent monomodified aminoferrocenes are available for these cells,[19] allowing comparisons to be made between the known and new prodrugs. Doubly modified prodrugs **2a** (IC_{50} = 20 ± 1 μM) and especially **2b** (IC_{50} = 14 ± 5 μM) were found to be more toxic to HL-60 cells than the parent prodrug **1a** (IC_{50} = 52 ± 3 μM). This was the result that we expected because we initially assumed that both boronic acid residues could be cleaved from **2a** and **2b** under the cancer-specific conditions (high concentration of H_2O_2), thereby inducing the formation of 2 eqquiv of *p*-quinone methide **8** and 1 equiv of iron ions per 1 equiv of prodrug, as shown in inset A of Scheme 1. In contrast, monofunctional prodrugs can generate only 1 equiv of *p*-quinone methide **8** and 1 equiv of iron ions. However, the spectroscopic and mass spectrometric data described above indicate that only one boronic acid residue is cleaved in the presence of cancer-specific H_2O_2 concentrations (<100 μM) from **2a**, **2b**, and **1a**. We found that the toxicity trend correlates with the membrane permeability of the prodrugs rather than with their reactivity. In particular, **2a** was found to be 2.3-fold and **2b**, 4.1-fold more membrane permeable than **1a** (Table 2).

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Table 2. Cellular Membrane Permeability, Efficacy of ROS Release, and Toxicity of Aminoferrocene-Based Prodrugs and Control Compounds towards Human Promyelocytic Leukemia Cells (HL-60)

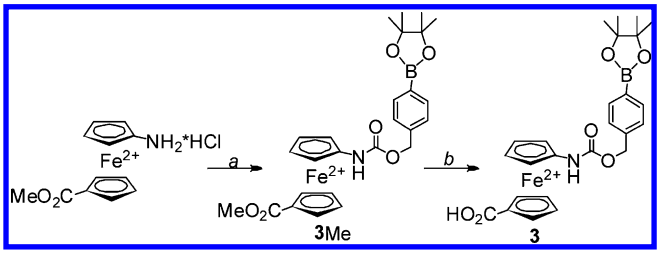
entry	drug	membrane permeability	efficacy of ROS release[a]	IC_{50} (μM)[b]
1	**1a**	1.0	1.0	52 ± 3
2	**2a**	2.3 ± 0.7	1.3 ± 0.2	20 ± 1
3	**2b**	4.1 ± 1.8	[c]	14 ± 5
4	**3**	0.4 + 0.1	0.3 ± 0.1	68 ± 15
5	**1e**	1.8 ± 0.3	1.6 ± 0.4	9 ± 2
6	**4a**	1.2 ± 0.2	1.1 ± 0.3	12 ± 1
7	**4b**	3.4 ± 2.5	1.1 ± 0.3	21 ± 4
8	**6**		0.1 ± 0.1	>200

[a]Efficacy of ROS release was defined as F/F(**1a**), where F is the mean fluorescence of the cells (λ_{ex}= 488 nm and λ_{em}= 530 nm, determined by flow cytometry) loaded with DCDFH (10 μM) and treated with prodrug (100 μM) for 4.5 h. The mean fluorescence obtained for prodrug **1a** was used as a reference (1.0). [b]IC_{50} values were determined using a propidium iodide-based assay.[19] [c]An accurate determination of ROS-release was not possible because of the high toxicity of prodrug **2b** under the experimental conditions employed.

In agreement with the higher toxicity and membrane permeability of **2a**, the former prodrug induces the generation of 1.3-fold more ROS than **1a** in cells (entry 3, Table 2). Unfortunately, we could not accurately determine the ROS amount released in cells treated with **2b** because this compound is too toxic under the experimental conditions applied.

1-Amino-1′-carboxyferrocene-Based Prodrug. Aminoferrocene-based prodrugs prepared earlier[19] are not very soluble in aqueous solutions buffered at pH 7. Therefore, all assays were conducted in solutions containing 0.1−1% cosolvent(e.g., *N*,*N*-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO)). To improve the solubility of the prodrugs, we introduced a carboxylic acid substituent into parent structure **1a** to obtain prodrug **3**. The latter compound was synthesized starting from commercially available 1-amino-1′-methoxycarbonylferrocene hydrochloride, which was first converted to 1-isocyanato-1′-methoxycarbonyl-ferrocene by neutralization with triethylamine followed by the reaction with triphosgene. The isocyanate and 4-(hydroxymethyl)-phenylboronic acid pinacol ester were then coupled together to obtain the methyl ester of the prodrug (**3Me**, Scheme 3). The last step of the synthesis included the hydrolysis of the ester, which was optimized to minimize the possible side reaction of the cleavage of the boronic acid ester fragment in **3Me**. In particular, the following conditions were tested: NaOH in THF/water, LiOH in MeOH/H_2O,[23] Lipase B from *Candida antarctika* immobilized on acrylic resign,[24] and LiI in 2,6-lutidine.[25] The highest yield of the desired product (39%) was obtained under Corey's conditions (LiOH).

Scheme 3. Synthesis of Water-Soluble Prodrug 3[a]



[a](a) (1) NEt_3; (2) triphosgene, toluene and (b) LiOH, MeOH, H_2O.

Although the solubility of prodrug **3** in aqueous buffered solution was improved with respect to that of **1a** and the in vitro iron- and ROS-releasing properties of **3** were found to be favorable (entries 1 and 4, Table 1), the effects of **3** in cells were substantially weaker than those of **1a** (entries 1 and 4, Table 2). In particular, the former prodrug generates 3.3 times less ROS in HL-60 cells than **1a** and correspondingly its toxicity toward HL-60 cells is lower (IC_{50} = 68 vs 52 μM for **1a**). The diminished activity of **3** in cells could be explained by its low cell-membrane permeability (entry 4, Table 2), which is probably caused by its negative charge at physiological pH. Therefore, overall, the attachment of the carboxylic acid function has a negative influence on the properties of aminoferrocene-based prodrugs. We are currently using the carboxylic acid group in compound **3** as an anchor to attach variable structural fragments to obtain aminoferrocene-based prodrugs with improved water solubility, cancer cell targeting, and reactivity toward ROS.

***N*-Benzylaminoferrocene-Based Prodrugs.** From the compounds that we described in our first report on aminoferrocene-based prodrugs (Scheme 1),[19] *N*-benzyl-substituted prodrug **1e** exhibited the highest activity against HL-60 cells (IC_{50} = 9 μM) and excellent cancer-cell selectivity. On the basis of the experimental data, we concluded that this high activity was both because of the formation in cancer cells of a relatively stable, ROS-generating catalyst *N*-benzylaminoferrocene and *p*-quinone methide and because of the high cell-membrane permeability of the prodrug. In an attempt to improve further the cell-membrane permeability of these compounds, we prepared more hydrophobic prodrugs **4a** and **4b** containing 4-methyl and 4-ethyl substituents at the *N*-benzyl residue. These compounds were prepared analogously to **1e** except that 4-methylbenzaldehyde (in the synthesis of **4a**) or 4-ethylbenzaldehyde (in the synthesis of **4b**) were used in place of benzaldehyde. In contrast to our expectations, the cell-membrane permeability of parent compound **1e** was slightly higher than that of *p*-methyl-substituted prodrug **4a** (entries 5 and 6, Table 2). Moreover, although the permeability of *p*-ethyl-substituted prodrug **4b** was found to be better than that of **1e**, the large standard deviation of this parameter (±2.5) does not allow for its accurate comparison with the permeability of other prodrugs. Furthermore, the ROS-generation in the presence of **1e** was found to be more efficient than that in the presence of either **4a** or **4b** (entries 5−7, Table 1). We speculate that deactivation of the latter prodrugs can be caused by their aggregation in aqueous solution. In agreement with the in vitro properties of **4a** and **4b**, their toxicity toward HL-60 cells and their ROS-generation ability in these cells is diminished with respect to those of **1e** (entries 5−7, Table 2). On the basis of these data, we can conclude that the introduction of alkyl substituents at the para-position of the *N*-benzyl fragment does not improve the anticancer properties of the aminoferrocene-based prodrugs.

It should be mentioned that within the experimental error the toxicities of all newly and earlier prepared aminoferrocene-based prodrugs correlate with the efficiency of ROS-generation in cells, as shown in the plot in Figure 2.

These data may indicate that all aminoferrocene-based anticancer prodrugs studied to date act via a related mechanism that relies on the catalytic generation of ROS in cancer cells.

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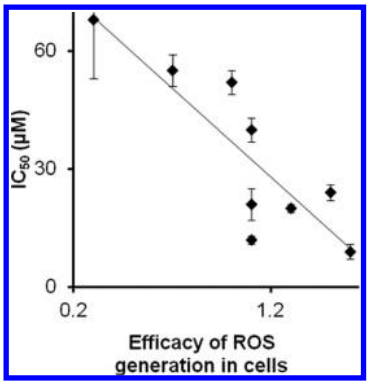

Figure 2. Correlation of the toxicity (IC_{50}, μM) of the known[19] and described in this Article aminoferrocene-based prodrugs with their efficiency in the generation of ROS in cells. The efficiency of ROS generation is expressed in relative units (r.u.), which was determined relative to the efficiency obtained for prodrug **1a**.

However, more experimental data are required to confirm this suggestion.

Activity of Aminoferrocene-Based Prodrugs toward Chronic Lymphocytic Leukemia (CLL) and Human Mononuclear Cells (MNC). As described above and in our previous publications,[19] aminoferrocene-based prodrugs are toxic toward a variety of cancer cell lines. As the next step in the development of these compounds, we decided to evaluate their activity toward primary cancer cells, which are isolated directly from cancer patients. We selected CLL cells as an example of cancer cells and MNC's as an example of normal cells. MNC's as well as CLL cells were both isolated from peripheral blood. Cancerous CLL cells are derived from B-cells, which, together with T-cells and monocytes, are present in MNC's. Therefore, the CLL and MNC pair is a good model to study the cancer specificity of new drugs. Another reason for the selection of CLL was the fact that intracellular ROS concentrations in these cells were reported to be increased. Finally, CLL responds to As_2O_3 treatment, which is known to increase the ROS amount in cells and thereby cause their death.[26] Therefore, it was sensible to assume that our ROS-modulating aminoferrocene-based prodrugs will be efficiently activated in CLL cells.

CLL cells were isolated from four patients, whereas MNC's were isolated from six healthy donors. The toxicity of selected aminoferrocene-based prodrugs at five different concentrations in the range between 1 and 10 μM was evaluated using the ATP-based CellTiter Glo Luminescence Cell Viability Assay. These data were used to estimate the IC_{50} values and cancer specificity of the prodrugs (Table 3). In particular, we observed that the aminoferrocene-based prodrugs were not toxic toward MNC's at concentrations $\leq$10 μM, whereas their toxicity toward CLL cells was significant, with IC_{50} values in the low-micromolar range (1.4–6.0 μM) (Table 3). As expected, stable ferrocenes **6** and Fc exhibit toxicity neither toward normal (MNC) nor cancerous cells (CLL), whereas positive control **5**, which is activated nonspecifically both in normal and cancer cells because of intracellular esterase activity, exhibits significant toxicity toward both cells types (Table 3). To quantify and compare the effect of the prodrugs toward cancer and normal cells, we defined the cancer-cell-specificity factor as a ratio of the number of viable MNC's to the number of viable CLL cells that were treated with a prodrug of a particular concentration (Table 3). This factor was found to exceed 10 for aminoferrocenes **1e**, **2a**, and **2b** at a concentration of 5 μM and for **1a**, **1b**, **1e**, **2a**, and **2b** at a concentration of 7.5 μM (Figure 3).

Table 3. Toxicity of Aminoferrocene-Based Prodrugs towards Chronic Lymphocytic Leukemia (CLL) Cells and Mononuclear Cells (MNC)

	IC_{50} ± SD (μM)[a]		number of viable MNC/number of viable CLL cells	
prodrug	CLL	MNC	5 μM prodrug	7.5 μM prodrug
1a	2.2 ± 2.1	>10	7.2	14.5
1b	2.8 ± 2.2	>10	5.3	12.0
1c	3.9 ± 2.1	>10	3.3	6.5
1e[c]	1.5 ± 2.2	>10	11.1	12.5
2a[c]	1.8 ± 2.1	>10	10.3	12.3
2b[c]	1.4 ± 2.1	>10	12.4	14.8
3	6.0 ± 2.1	>10	1.6	2.5
4a	2.6 ± 2.2	>10	3.8	8.2
4b	3.1 ± 2.1	>10	4.1	9.8
5	1.0 ± 2.0	5.5 ± 2.6	4.5	0.9
6	>10	>10	1.3	1.3
Fc[b]	>10	>10	1.0	1.0

[a]SD, standard deviation. [b]Fc, ferrocene. [c]Indicates the prodrugs that exhibit most favorable properties.

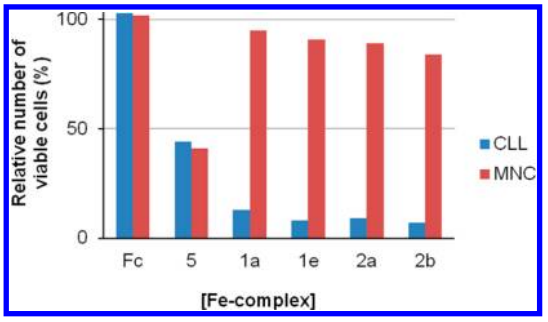

Figure 3. Relative viability of CLL cells and MNC's expressed as a percent of the control (untreated cells) treated with different prodrugs (7.5 μM) for 48 h.

The highest specificity of 14.8 was observed for prodrug **2b** at a concentration of 7.5 μM. In contrast, the related catalytic organochalcogene-based prodrugs, which were prepared and investigated by the research groups of C. Jacob and M. Herling, exhibited a cancer-cell specificity (CLL/MNC) that did not exceed 4 in similar assay.[15] Moreover, although the latter compounds exhibited significant anticancer activity at a somewhat lower concentration (0.5 μM) than our best prodrugs, **1e**, **2a**, and **2b** (IC_{50} = 1.4–1.8 μM and selectivity = 10.3–14.8, Table 3), their toxicity toward normal cells (MNC's) was also found to be higher.

To explore the toxicity of aminoferrocene-based prodrugs toward normal cells (MNC's) in more detail, we have studied the effects of a representative prodrug, **1e**, on MNC's at a high concentration (10 μM) and at longer incubation times (48–72 h). We observed that 76 ± 8% of viable MNC's survive 48 h of incubation in the presence of this prodrug, whereas a 72 h incubation causes a further reduction in the number of viable normal cells by a factor of 2. These data indicate that at high concentrations and prolonged incubation times aminoferrocene-based prodrugs can be also activated in normal cells, which are known to contain low amounts of reactive-oxygen species.

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Effects of Selected Aminoferrocene-Based Prodrugs on Bacteria. Some bacteria are important for the normal function of the human body. For example, harmless strains of *E. coli* are found in the gut of all healthy humans and assist the body, for example, by protecting it from other bacteria. It is important that new drugs/prodrugs do not affect these bacteria. Because bacteria are known to accumulate metal ions like iron, they can exist under a higher ROS load than normal cells of the human body. Therefore, bacteria can be potentially affected by the aminoferrocene-based prodrugs, which would be an undesired side effect. To test whether this is indeed the case, we investigated the toxicity of several selected prodrugs on Gram-negative *E.coli*: **1a**, **1e**, and **2a** were tested as well as a positive control, ampicillin, and a negative control, ferrocene. We observed that only ampicillin at 0.9 mM caused an inhibitory effect. In particular, a zone of inhibition was observed only for this compound (Figure S1, Supporting Information). In contrast, the bacteria were resistant to prodrugs **1a**, **1e**, and **2a** as well as the negative control at concentrations of ≤0.9 mM. Higher concentrations were not tested because of limited solubility. Additionally, we tested the same group of prodrugs and controls on *S. agalactiae*, which are Gram-positive bacteria present in the normal gastrointestinal flora of some humans. Consistent with the former experiments, we observed that the prodrugs are not active at concentrations of up to 0.9 mM.

CONCLUSIONS

We explored the effects of a few structural modifications of aminoferrocene-based prodrugs. In particular, we found that the attachment of a carboxylic acid substituent at the 1′-position of the ferrocene fragment improves the water solubility of the prodrug. However, it becomes less cell-membrane permeable. The synthesized carboxylic acid group-containing prodrug can be potentially applied as an intermediate for the further conjugation of fragments to improve water solubility, cancer targeting and specificity, and membrane permeability. The introduction of alkyl substituents at the para position of the *N*-benzylic residue of the corresponding N-substituted aminoferrocene-based prodrug does not improve the anticancer effect. In contrast, the attachment of the second arylboronic acid ester fragment to the parent prodrug enhances its activity significantly, from IC_{50} = 52 μM to 14−20 μM. Moreover, we demonstrated that the aminoferrocene-based prodrugs are active not only toward cancer cell lines but also toward primary cancer cells (CLL; IC_{50} = 1.4−1.8 μM for the best prodrugs). Importantly, they practically do not affect the corresponding normal cells (mononuclear cells, MNC) at concentrations <7.5 μM and incubation times ≤48 h. We estimated that the cancer-cell specificity reaches 14.8-fold in the best case. However, at high concentrations (10 μM) and prolonged incubation times (72 h), aminoferrocene-based prodrugs can be also activated in normal cells, which are known to contain low amounts of reactive-oxygen species. Finally, we observed that representative bacterial cells, including *E.coli* and *S. agalactiae*, which populate the gastrointestinal flora of humans and are required for the normal function of the human body, are not affected by the aminoferrocene-based prodrugs at concentrations up to 0.9 mM. These data are indicative of the high cancer specificity of these prodrugs, which can potentially make them suitable for in vivo applications.

EXPERIMENTAL SECTION

General Information. Commercially available chemicals of the best quality from Aldrich/Sigma/Fluka (Germany) were obtained and used without purification. Prodrugs **1a** and **1e** as well as control **6** were prepared as described previously.[25] NMR spectra were acquired on a Bruker Avance DRX 200, Bruker Avance II 400, or Bruker Avance III 600 spectrometer. ESI mass spectra were recorded on an ESI MicroTOF (Bruker), FAB mass spectra, on a Jeol JMS-700 instrument using *p*-nitrobenzyl alcohol as a matrix, and EI mass spectra, on a Finnigan MAT 8200 instrument. C, H, and N analysis was performed in the microanalytical laboratory of the chemical institute of the University of Heidelberg. For analytical reversed-phase thin-layer chromatography, Polygramm TLC plates (Macherey-Nagel) were used. UV−vis spectra were acquired on a Varian Cary 100 Bio UV−vis spectrophotometer using 1 cm optical path black-wall absorption semimicrocuvettes (Hellma GmbH, Germany) with a sample volume of 0.7 mL. Fluorescence spectra were acquired on a Varian Cary Eclipse fluorescence spectrophotometer using black-wall fluorescence semimicrocuvettes (Hellma GmbH) with a sample volume of 0.7 mL. The fluorescence of live HL-60 cells was quantified using an Accuri C6 flow cytometer. The data were processed using the CFLow Plus (Accuri) software package. 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%.

Synthesis. *1,1′-Bis(azidocarbonyl)ferrocene.*[22] 1,1-Ferrocenedicarboxylic acid[21] (6.0 g, 21.9 mmol) was suspended in CH_2Cl_2 (35 mL) and purged with argon. Oxalylchloride (11.2 g, 88.2 mmol, 4.5 equiv) and *N*,*N*-dimethylformamide (DMF, 15 mL) were then added, and the reaction mixture was mixed for 3 h at 22 °C. During this time, two new portions of oxalylchloride (2 × 1 g, 15.8 mmol, 0.5 equiv) were added at 1 and 2 h after the beginning of the reaction. Excess oxalylchloride and solvents were removed under vacuum (0.01 mbar), and the residue was dissolved in CH_2Cl_2 (140 mL) and mixed with $(n\text{-}Bu)_4NBr$ (17.9 g, 55.6 mmol, 2.5 equiv). A solution of NaN_3 (4.8 g, 73.1 mmol, 3.3 equiv) in water (40 mL) was then added dropwise, and the resulting mixture was stirred for a further 18 h at 22 °C. Next, water (100 mL) was added, the organic phase was separated, and the aqueous phase was washed with CH_2Cl_2 (3 × 20 mL). The organic phases were joined together, dried under $MgSO_4$, and filtered. Finally, the solvent was removed under vacuum using a rotary evaporator, and the product was purified by column chromatography on SiO_2 using CH_2Cl_2 as an eluent. The yield of the product was 4.6 g (65%). TLC (SiO_2, CH_2Cl_2 eluent) R_f = 0.50; ^{1}H NMR ($CDCl_3$, 399.89 MHz) δ 4.56 (s, 4H), 4.90 (s, 4H); EI−MS, pos, *m*/*z*: calcd for $[C_{12}H_8N_6O_2Fe\text{-}e^-]^+$, 324.0; found, 324.2

Prodrug ***2a.*** 1,1′-Bis(azidocarbonyl)ferrocene (0.8 g, 2.5 mmol) was dissolved in toluene (150 mL) and purged with argon. The solution was heated to reflux and kept under these conditions for 2.5 h to obtain 1,1′-diisocyanatoferrocene. Next, the solution was cooled to 22 °C, 4-(hydroxymethyl)phenyl-boronic acid pinacol ester (1.3 g, 5.7 mmol, 2.3 equiv) in CH_2Cl_2 (80 mL) was added, and the resulting mixture was stirred for 22 h at 22 °C. Finally, the volatiles were removed using a rotary evaporator, and the crude product was purified by column chromatography on SiO_2 using petroleum ether/ethyl-acetate mixture (2:1 v/v) as an eluent. The resulting oil was resuspended in ethylacetate, and the solid product formed was filtered and dried. The yield of the analytically pure product was 162 mg (9%). TLC (SiO_2, *n*-hexane/ethyl acetate 2:1 v/v eluent) R_f = 0.38; ^{1}H NMR (DMSO-d_6, 399.89 MHz) δ 1.28 (s, 24H), 3.86 (s, 4H), 4.40 (s, 4H), 5.08 (s, 4H), 7.36 (d, ^3J = 7.3 Hz, 4H), 7.66 (d,^3J = 7.8 Hz, 4H), 8.83 (s, 1H); ^{13}C NMR (DMSO-d_6, 150.90 MHz) δ 24.83, 59.93, 61.06, 64.81, 65.29, 83.84, 126.95, 130.18, 134.06, 138.62, 140.51, 153.71; FAB−MS, pos, *m*/*z*: calcd for $[C_{38}H_{46}N_2O_8B_2Fe\text{-}e^-]^+$, 736.3; found, 736.5; Anal. Calcd for $C_{38}H_{46}N_2O_8B_2Fe$: C, 61.99; H, 6.30; N, 3.80. Found: C, 61.79; H, 6.38; N, 3.61.

Prodrug ***2b.*** This compound was synthesized analogously to that of prodrug **2a** except that 4-(hydroxymethyl)-2-methylphenyl-boronic acid pinacol ester was used in place of 4-(hydroxymethyl)-

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phenylboronic acid pinacol ester as a starting material. The yield of the analytically pure product was 185 mg (18%). TLC (SiO_2, *n*-hexane/ethyl acetate 2:1 v/v eluent) R_f = 0.48; ^{1}H NMR (acetone-d_6, 600.13 MHz) δ 7.85 (bs, 1H), 7.74−7.69 (d, ^3J = 7.4 Hz, 2H), 7.18 (bs, 4H), 5.09 (s, 4H), 4.51 (s, 4H), 3.93, (s, 4H), 2.51 (s, 6H), 1.33 (s, 12H); ^{13}C NMR (acetone-d_6, 150.91 MHz) δ 154.93, 145.73, 140.81, 137.02, 129.72, 129.09, 124.74, 98.03, 84.26, 66.61, 65.65, 62.49, 25.21, 22.51; EI−MS, pos, *m*/*z*: calcd for $[C_{40}H_{50}N_2O_8B_2Fe\text{-}e^-]^+$, 764.3; found, 764.5; Anal. Calcd for $C_{40}H_{50}N_2O_8B_2Fe$·0.5 hexane: C, 63.97; H, 7.12; N, 3.47. Found: C, 63.71; H, 6.70; N, 3.04.

1-Amino-1′-methoxycarbonylferrocene. 1-Amino-1′-methoxycabonyl-ferrocene hydrochloride (2.0 g, 6.8 mmol) dissolved in ethylacetate (150 mL) was slowly neutralized with NEt_3 (2.4 mL, 17.0 mmol, 2.5 equiv). The resulting mixture was washed with water (10 × 30 mL), and the combined aqueous phases were washed with ethylacetate (2 × 30 mL). The organic phases were combined and dried over $MgSO_4$, the solvent was removed under vacuum (50 mbar) in a rotary evaporator, and the solid product was dried under vacuum (0.01 mbar) to obtain 1.8 g (99%) of the desired product. TLC (SiO_2, petroleum ether (30-75)/ethyl acetate/NEt_3 7.5:2.5:0.5 v/v/v eluent) R_f = 0.21; ^{1}H NMR (acetone-d_6, 399.89 MHz) δ 3.73 (s, 3H), 3.80 (t, 2H), 3.93 (t, 2H), 4.31 (t, 2H), 4.66 (t, 2H).

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-benzyloxycarbonylamino-ferrocene (Methyl Ester of Prodrug ***3****,* ***3Me****).* 1-Amino-1′-methoxy-carbonylferrocene (2.3 g, 9 mmol) was dissolved under Ar in toluene (100 mL). Triphosgene (2.4 g, 8 mmol, 0.9 equiv) was added, and the reaction mixture was heated to reflux and kept under these conditions for 90 min. Next, the mixture was allowed to cool to 22 °C, a solution of 4-(hydroxymethyl)-phenylboronic acid pinacol ester (1.9 g, 8 mmol, 0.9 equiv) in toluene (23 mL) was added, and the solution was mixed for 92 h at 22 °C. After the removal of the solvent using a rotary evaporator, the product was purified by column chromatography on SiO_2 using pethroleum ether (30−75)/ethylacetate initially as an eluent. The content of ethylacetate in this eluent was gradually increased during the chromatography up to 100%. The yield of the product was 3.7 g (79%). TLC (SiO_2, petroleum ether (30-75)/ethyl acetate 3:1 v/v eluent) R_f = 0.28; ^{1}H NMR (acetone-d_6, 399.89 MHz) δ 1.33 (s, 12H), 3.70 (s, 3H), 3.99 (s, 2H), 4.38 (s, 2H), 4.66 (s, 2H), 4.73 (s, 2H), 5.19 (s, 2H), 7.44 (d, 2H), 7.76 (d, 2H), 8.05 (s, 1H).

Prodrug ***3****.* Methyl ester **3Me** (2.0 g, 3.9 mmol) dissolved in MeOH (250 mL) was mixed with solution of LiOH (2.6 g, 62 mmol, 16.1 equiv) in water (53 mL), and the reaction mixture was stirred at 22 °C for 90 min. After the removal of the solvent under vacuum, the remaining solid was suspended in a solvent mixture consisting of ethylacetate (250 mL) and acetic acid (4 mL). This mixture was washed with water (20 mL), and the solvent was removed using a rotary evaporator. The product was purified by column chromatography on SiO_2 using pethroleum ether (30-75)/ethylacetate (7:1 v/v) initially as an eluent followed by pethroleum ether (30−75)/ethylacetate (3:1 v/v) and pethroleum ether (30-75)/ethylacetate (3:1 v/v) containing 0.5% acetic acid as eluents. The yield of the product was 0.8 g (39%). TLC (SiO_2, petroleum ether (30−75)/ethyl acetate 1:1 v/v containing 5% acetic acid eluent) R_f = 0.58; ^{1}H NMR (acetone-d_6, 600.13 MHz) δ 1.33 (s, 12H), 4.00 (s, 3H), 4.38 (s, 2H), 4.65 (s, 2H), 4.73 (s, 2H), 5.18 (s, 2H), 7.43 (d, 2H), 7.75 (d, 2H), 8.09 (s, 1H); ^{13}C NMR (acetone-d_6, 150.90 MHz) δ 24.43, 61.17, 65.68, 65.78, 70.93, 72.23, 72.37, 83.67, 98.41, 126.77, 134.53, 134.71, 140.41, 153.67, 171.19; FAB−MS, pos, *m*/*z*: calcd for $[C_{25}H_{28}NO_6BFe\text{-}e^-]^+$, 505.1; found, 505.3; Anal. Calcd for $C_{25}H_{28}NO_6BFe$·1/3 acetic acid: C, 58.70; H, 5.63; N, 2.67. Found: C, 58.85; H, 5.68; N, 2.59.

Prodrug ***4a****.* Aminoferrocene (0.40 g, 2 mmol), 4-methylbenzaldehyde (0.24 g, 2 mmol), and MeOH (10 mL) were mixed and brought to reflux. The reagents were allowed to react for 2 h under these conditions. The mixture was allowed to cool to 22 °C, $NaB(CN)H_3$ (0.13 g, 2 mmol) in MeOH (10 mL) was added, and the mixture was stirred for 30 min. Next, aqueous HCl (1 M, 2 mL) was slowly added, and the volatiles were removed using a rotary evaporator and then under vacuum (0.01 mbar). The residue was resuspended in toluene (7 mL), triphosgene (bis(trichloromethyl)carbonate, 0.60 g, 2 mmol) was added, and the mixture was heated to reflux and kept under these conditions for 1.5 h. The solution obtained was cooled to 22 °C, and 4-(hydroxymethyl)phenylboronic acid pinacol ester (0.46 g, 2 mmol) in toluene (5 mL) was added. The mixture was again heated to reflux and left stirring under these conditions for 15 h. After cooling to 22 °C, more triphosgene (1.2 g, 4 mmol) was added, and the mixture was stirred at 22 °C for a further 20 h. Finally, all volatiles were removed under vacuum (0.01 mbar), and the residue was purified by column chromatography on SiO_2 using *n*-hexane/acetone mixture (95:5 v/v) as an eluent. The yield of the analytically pure product was 0.19 g (17%). TLC (SiO_2, *n*-hexane/acetone 3:1 v/v eluent) R_f = 0.5; ^{1}H NMR (acetone-d_6, 399.89 MHz) δ 1.33 (s, 12H), 2.31 (s, 3H), 3.97 (s, 2H), 4.11 (s, 5H), 4.46 (s, 2H), 4.96 (s, 2H), 5.24 (s, 2H), 7.18 (m, 4H), 7.38 (m, 2H), 7.73 (d, ^3J = 7.0 Hz, 2H); ^{13}C NMR (acetone-d_6, 100.55 MHz) δ 21.14, 25.27, 53.88, 63.27, 65.09, 67.84, 69.81, 84.64, 127.35, 129.99 (2 overlapping peaks), 135.66 (2 overlapping peaks), 136.89, 137.20, 141.00; FAB−MS, pos, *m*/*z*: calcd for $[C_{32}H_{36}NO_4BFe\text{-}e^-]^+$, 565.2; found, 565.2; Anal. Calcd for $C_{32}H_{36}NO_4BFe$: C, 67.99; H, 6.42; N, 2.48. Found: C, 68.34; H, 6.87; N, 2.38.

Prodrug ***4b****.* This compound was synthesized analogously to that of prodrug **4a** except that 4-ethylbenzaldehyde was used in place of 4-methylbenzaldehyde as a starting material. The yield of the analytically pure product was 78 mg (13%). TLC (SiO_2, *n*-hexane/acetone 5:1 v/v eluent) R_f = 0.47; ^{1}H NMR (acetone-d_6, 399.89 MHz) δ 7.74 (d, ^3J = 7.8 Hz, 2H), 7.38 (d, ^3J = 7.9 Hz, 2H), 7.20 (m, 4H), 5.24 (s, 2H), 4.97 (s, 2H), 4.45 (bs, 2H), 4.11 (s, 5H), 3.96 (s, 2H), 2.62 (q, ^3J = 7.5 Hz, 2H), 1.33 (s, 12H), 1.21 (t, ^3J = 7.5 Hz, 3H); ^{13}C NMR (acetone-d_6, 100.55 MHz) δ 143.71, 140.97, 137.12, 135.65, 128.88, 128.81, 128.01, 127.83, 127.41, 84.62, 69.78, 67.84, 67.59, 65.07, 63.27, 53.86, 29.13, 25.27, 16.24; ESI−MS, pos, *m*/*z*: calcd for $[C_{33}H_{38}NO_4BFe\text{-}e^-]^+$, 579.2; found, 578.8; Anal. Calcd for $C_{33}H_{38}NO_4BFe$·1.5 hexane: C, 71.19; H, 8.39; N, 1.98. Found: C, 71.12; H, 8.19; N, 2.14.

4-(Hydroxymethyl)phenylacetate. 4-Acetoxybenzoic acid (2.5 g, 13.9 mmol) was suspended in tetrahydrofurane (THF, 18 mL) and cooled to 10 °C. A solution of BH_3·THF complex in THF (1 M, 30 mL, 30 mmol) was added dropwise over 10 min time. The mixture was allowed to warm to 22 °C and was left stirring under these conditions for 36 h. The mixture was then cooled to 0 °C, and water (11 mL) was slowly added. After the removal of the solvent under vacuum (0.01 mbar), the residue was resuspended in a water/ethyl acetate mixture (140 mL, 1:1 v/v), the organic phase was separated and washed with water (70 mL), a saturated aqueous NaCl solution (3 × 70 mL), and again with water (2 × 70 mL), dried under Na_2SO_4, and filtered. Finally, the solvent was removed using a rotary evaporator, and the residue was purified by column chromatography on SiO_2 using a CH_2Cl_2/ethyl acetate/triethylamine (TEA) mixture (10:4:0.5 v/v/v) as an eluent. The yield of the product was 0.9 g (41%). TLC (SiO_2, CH_2Cl_2/ethyl acetate/TEA 10:4:0.5 v/v/v eluent) R_f = 0.41; ^{1}H NMR (acetone-d_6, 199.92 MHz) δ 2.24 (s, 3H), 4.21 (t, ^3J = 5.8 Hz, 1H), 4.62 (d, ^3J = 5.6 Hz, 2H), 7.06 (d, ^3J = 8.6 Hz, 2H), 7.38 (d, ^3J = 8.7 Hz, 2H).

4-(N-(Ferrocenylamino)carbonyloxymethyl)phenylacetate, Control ***5****.* Aminoferrocene (0.4 g, 2 mmol) and triphosgene (0.59 g, 2 mmol) in toluene (25 mL) were heated to reflux and kept under these conditions for 1 h. This led to dissolution of all reagents. Afterwords, the mixture was allowed to cool to 22 °C, 4-(hydroxymethyl)phenyl acetate (0.33 g, 2 mmol) in toluene (25 mL) was added, and the resulting mixture was stirred for 70 h at 22 °C. Finally, the volatiles were removed using a rotary evaporator, and the crude product was purified by column chromatography on SiO_2 using petroleum a ether (30-75)/ethylacetate mixture (7:1 to 7:3 v/v) as an eluent. The product was washed several times with *n*-hexane and dried. The yield of the pure product was 0.11 g (14%). TLC (SiO_2, *n*-hexane/acetone 7:3 v/v eluent) R_f = 0.37; ^{1}H NMR ($CDCl_3$, 399.89 MHz) δ 2.31 (s, 3H), 4.00 (s, 2H), 4.17 (s, 5H), 4.50 (s, 2H), 5.15 (s, 2H), 5.88 (s, 1H), 7.11 (d, ^3J = 8.4 Hz, 2H), 7.42 (d, ^3J = 8.4 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100.55 MHz) δ 21.11, 60.93, 64.51, 66.35, 69.18, 77.20, 121.74, 129.45, 133.83, 150.56, 169.39; FAB−MS, pos, *m*/*z*: calcd for

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$[C_{20}H_{19}NO_4Fe\text{-}e^-]^+$, 393.1; found, 393.0; Anal. Calcd for $C_{20}H_{19}NO_4Fe$·0.5 ethylacetate: C, 60.43; H, 5.30; N, 3.20. Found: C, 60.54; H, 4.86; N, 3.38.

Cellular Assays. *Cells and Cell Culture.* The human promyelocytic leukemia cell line (HL-60) and primary CLL and MNC's were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FCS and 5 μg/mL of penicillin/streptomycin (all media and supplements were from Gibco Invitrogen Corp., Karlsruhe, Germany).

ROS Detection in Cells and Determination of the Viability of the Cells. Intracellular ROS and the viability of HL-60 cells were monitored using flow cytometry as described previously.[19] The viability of CLL cells and MNC's was determined by CellTiter Glo Luminescence Cell Viability Assay (Promega, Fitchburg, USA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Protocols and data for the effects of prodrugs and controls on *E. coli* and *S. agalactiae*. Protocols for determining the octanol–water partition coefficients and membrane permeability of aminoferrocene-based prodrugs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

[‖]The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. These authors contributed equally.

Notes

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■ ABBREVIATIONS USED

bipy, 2,2-bipyridine; BSO, buthionine sulfoximine; CH2Cl2, dichloromethane; CLL, chronic lymphocytic leukemia; DCDFH, 2′,7′-dichlorodihydrofluorescein; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DNP, 2,4-dinitrophenol; EDTA, *N*,*N*,*N*′,*N*′-ethylenediamine tetraacetic acid; Fc, ferrocene; FCCP, *p*-trifluoromethoxyphenylhydra-zone; FCS, fetal bovine serum; *E. coli*, *Escherichia coli*; GSH, reduced glutathione; GSSG, oxidized glutathione; HL-60, human promyelocytic leukemia cells; 8-HQ, 8-hydroxyquinoline; 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; QM, quinone methide; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; *S. agalactiae*, *Streptococcus agalactiae*; TLC, thin layer chromatography

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