An Endoplasmic Reticulum Specific Pro-amplifier of Reactive Oxygen Species in Cancer Cells

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**Abstract:** The folding and export of proteins and hydrolysis of unfolded proteins are disbalanced in the endoplasmic reticulum (ER) of cancer cells, leading to so-called ER stress. Agents further augmenting this effect are used as anticancer drugs including clinically approved proteasome inhibitors bortezomib and carfilzomib. However, these drugs can affect normal cells, which also rely strongly on ER functions, leading, for example, to accumulation of reactive oxygen species (ROS). To address this problem, we have developed ER-targeted prodrugs activated only in cancer cells in the presence of elevated ROS amounts. These compounds are conjugates of cholic acid with N-alkylaminoferrocene-based prodrugs. We confirmed their accumulation in the ER of cancer cells, their anticancer efficacy, and cancer cell specificity. These prodrugs induce ER stress, attenuate mitochondrial membrane potential, and generate mitochondrial ROS leading to cell death via necrosis. We also demonstrated that the new prodrugs are activated in vivo in Nemeth-Kellner lymphoma (NK/Ly) murine model.

According to estimation of European Cancer Information System (ECIS) in the European Union 1.3 millions of people will die from cancer and over 2.7 million of new cases (excluding non-melanoma skin cancer) will be diagnosed in 2020. Treatment of this disease is complicated, since cancer and normal cells are related and, correspondingly, precise targeting/killing of the former cells without affecting of the latter ones is a challenging and not yet fully solved problem. In particular, currently available, clinically approved chemotherapeutics exhibit dose-limiting side effects, which adversely and sometimes irreversibly affect the quality of life of patients. Cancer cells can be addressed specifically by making use of their unique features, for example, the presence of some overexpressed receptors, altered glycolysis, and elevated amounts of reactive oxygen species (ROS = \( \text{H}_2\text{O}_2, \text{O}^{\cdot-} \), and HO\(^{\cdot-} \)). The endoplasmic reticulum (ER) has been recognized as an especially attractive target due to the following reasons. In cancer cells the balance between folding/transport of proteins and degradation of misfolded proteins is disturbed that leads to the so-called ER stress. Further potentiation of the stress by chemical agents induces cancer cell death, whereas normal cells initially lacking the ER stress withstand the drug effect. Though some cancer cell selectivity can be achieved, side effects are also expected. They include, but not limited to induction of moderate ER stress in normal cells leading to unfolded protein response (UPR), elevation of intracellular amounts of ROS and disbalance of Ca\(^{2+} \) homeostasis. All these factors contribute to genome instability that stimulate carcinogenesis.

Herein we addressed this problem by the development of ER targeting prodrugs \( 4a-g \), which are activated only in cancer cells, but remain inactive in normal cells (Scheme 1, Scheme 2). These compounds are based on N-substituted aminoferrocene (AF) drugs. After their formation in cells these drugs cycle between reduced (AF) and oxidized (AF\(^{+} \)) forms catalyzing the formation of highly reactive ROS \( \text{O}_2^{\cdot-} \) (from \( \text{O}_2 \)) and HO\(^{\cdot-} \) (from \( \text{H}_2\text{O}_2 \)) that leads to cell death.
relies on the activation by binding of Cu ions in cells. Apart from inducing ER stress via proteasome inhibition and ROS production,\textsuperscript{[18]} it affects other targets including inhibition of aldehyde dehydrogenase (ALDH) and the STAT3 signaling pathway.\textsuperscript{[13]} Modulation of DNA-topoisomerases and methyltransferases as well as glutathione S-transferase.\textsuperscript{[18]} Thus, the known anticancer prodrugs are not ER specific.

Previously reported ER-carriers are usually hydrophobic structures, for example, cyanine dye DiOC6, hexyl rhodamine B, polyethylene glycol (PEG), long alkyl chains, derivatives of p-methylphenylsulfonylamides.\textsuperscript{[10]} Since water solubility of AF-prodrugs is limited (≤50 μM),\textsuperscript{[9,12–14]} their further modification with the known ER-carriers would with high probability lead to non-soluble in water compounds. Therefore, we searched for alternative modifiers. We selected bile acids, since along with their hydrophobic core, they carry polar groups (alcohol or carbonyl), which could provide for water solubility. To find the best modifier, we introduced a series of bile acid fragments (substituent R, Scheme 2) to obtain prodrugs 4a–4e as described in the supporting information (SI). We were pleased to observe that all prepared prodrugs are soluble at least up to 50 μM in aqueous solution (Table S1, Sf). All prodrugs affect the viability of Burkitt’s lymphoma BL-2 cells (Table S2, Sf) selected as a representative cancer cell line. Derivatives of cholic (4a, IC_{50} = 9 ± 2 μM) and dehydrocholic acid (4e, IC_{50} = 9 ± 2 μM) are most potent in this series. They exhibit a stronger anticancer effect than the non-targeted control 1\textsuperscript{[9]} (IC_{50} = 34 ± 3 μM, p < 0.001) and the Mit-targeting prodru 3\textsuperscript{[14]} (IC_{50} = 35 ± 2 μM, p < 0.001) reaching the potency of the best previously reported LY-targeting prodru 2\textsuperscript{[13]} (IC_{50} = 5 ± 2 μM). Based on these data and due to its easier synthesis, prodru 4a was selected for more detailed studies.

As previously established, prodrugs containing aryboronic acid pinacol ester are hydrolyzed in aqueous buffered at pH 7 solutions within 1 h.\textsuperscript{[20]} Therefore, the active form of 4a will be the boronic acid 4a-BA. We determined n-octanol/water partition coefficient (logP) of 4a-BA to be substantially higher (5.9 ± 0.2, Table S3, Sf) than that of parent prodru 1-BA (2.7 ± 0.2). The high lipophilicity of 4a-BA was expected to facilitate its accumulation in the ER.\textsuperscript{[19]}

To investigate the mechanism of 4a activation in the presence of H_{2}O_{2}, we applied electrospray ionization (ESI) mass spectrometry (MS). We confirmed that 4a is first hydrolyzed in aqueous solution forming 4a-BA. In the presence of H_{2}O_{2}, AF-drug 4a-BA (formed (Figures S29–S34, Sf), which can donate an electron to H_{2}O_{2}/O_{2}, leading to formation of ferrocenium 4a-2’ and highly toxic HO¯/O_{2}^-.

We confirmed experimentally formation of HO¯/O_{2}^- in mixtures of 4a and H_{2}O_{2} by using 2,7-dichlorodihydrofluorescein (DCFH) (Figure S35, Table S4, Sf). In particular, we found that 4a accelerates the rate of DCFH oxidation by 3.2-fold with respect to the rate of its spontaneous oxidation by H_{2}O_{2}. All together these data indicate that 4a is activated as outlined in Scheme 1 similarly to other known AF-prodrugs.\textsuperscript{[9,12–14]}

Further, we confirmed that apart from BL-2 cells prodru 4a exhibits anticancer activity towards other cancer cell lines including ovarian cancer A2780 (IC_{50} = 5.4 ± 0.7 μM) and T-...
cell leukemia Jurkat (IC_{50} = 18 ± 1 μM). The anticancer activity of LY-targeting prodrug 4a is similar towards A2780 cells (IC_{50} = 7 ± 2 μM) and higher towards Jurkat cells (IC_{50} = 7.2 ± 0.1 μM, p < 0.001), whereas that of Mit-targeting 3e is substantially lower for both cell lines (p < 0.001, Tables S5, S6, S7). For Jurkat cells we observed that 4a induces cell death mainly via necrosis and partially via apoptosis (Figure S36A, S7) that was reproduced for A2780 cells (Figure S37, S7). Since the direct anticancer effect of 4a will be facilitated by the action of immune system in vivo. We observed that the anticancer activity of 4a in Jurkat cells is decreased in the presence of the ROS inhibitor N-acetylcycteine (NAC)\[21\] (Figure S36B, S7). These data confirm that ROS is involved in the intracellular activation of 4a.

Next, we investigated the mechanism of action of 4a in cells. In the first experiment, we incubated A2780 cells with prodrug 4a as well as controls 1 and 2 for time periods between 1–24 h followed by their staining with organelle-specific dyes (ER: ER-tracker-green, ERT_{grn}; LY: acridine orange, AO; Golgi: Golgi-Staining-Green, GO; Mit: rhodamine 123, R123) and evaluation of their fluorescence by using flow cytometry (Figure 1). The fluorescence intensity of the cells treated with medium only (carrier) was used as a reference. We observed that non-targeted control 1 weakly affects the ER-specific fluorescence at 1 h incubation (but not at 4 h incubation) and does not affect the LY-, Golgi- and Mit-specific fluorescence at both 1 and 4 h incubation times. A weak decrease of the Mit-specific fluorescence was observed at the highest incubation time of 24 h. As expected, LY-targeted 2 strongly reduces the LY-specific fluorescence of the cells. Additionally, it also affects ER and especially Mit that can be a follow up effect after the initial lysosomal disruption as previously reported.\[13\] The effect of 4a on the ER-specific fluorescence of the cells for both 1 and 4 h incubation times was found to be strongest within the studied series of the prodrugs (p < 0.001, Figure 1A). Using confocal microscopy we observed that the ER tracker dye is leaking into the nuclei and the cytoplasm of a large proportion of 4a-treated cells indicating that 4a induces the ER disruption (Figure S38). In contrast to 2, prodrug 4a does not affect the LY-specific signal (Figure 1B), but at the early incubation time (1 h) decreases slightly the Golgi-specific fluorescence and does not affect the Mit-specific fluorescence. At the later incubation times the effects on Golgi and Mit become stronger. These data indicate that the ER is a primary site of action for 4a (the strong effect is seen already at 1h-incubation), whereas the effects on Golgi and Mit are secondary (≥4 h incubation is needed to observe strong effects).

By using quantitative PCR, we observed that the expression of mRNA of CCAAT-enhancer-binding protein homologous protein (CHOP), which is a marker of the ER stress,\[22\] is significantly increased in 4a-treated A2780 cells (p < 0.05). In contrast, neither unspecific 1 nor LY-targeting 2 affected the expression of the CHOP-mRNA (Table S7, S8). These data indicate that 4a exhibits its anticancer activity via the induction of the ER stress.

Next, we evaluated the ability of 4a to modulate the oxidative stress in cells (Figure 2A,B). We observed that this prodrug increases the total intracellular ROS amount (tROS)
in all studied cell lines significantly. However, the overall magnitude of the tROS increase was substantially lower than that found for LY-targeting 2[13]. In contrast, we observed the strong dose-dependent increase of mitochondrial ROS (mROS) in A2780 cells treated with 4a, whereas control 2 did not affect mROS at any concentration tested (0–30 μM). Thus, the mode of action of 4a (the induction of ER stress, the increase of mROS) is distinct from that implicated for all previously known AF-prodrugs[9,12–14,20].

To find out whether 4a is directly accumulated in ER or induces its effects on the ER indirectly, we prepared its fluorogenic analogue 4g containing a fluorescent dye 7-hydroxycoumarine (Sf). We confirmed that 4g and 4a are analogues. For example, they have similar solubility in aqueous solution (Table S1, SI), lipophilicity (Table S3, SI) and exhibit similar anticaner activity towards A2780 cells (IC50 = 6.2 ± 2.4 vs. IC50 = 5.4 ± 0.7 μM correspondingly).

Prodrug 4g is practically not fluorescent due to photo-induced electron transfer (PET) from the ferrocene moiety to the dye. Analogously to 4a, it is converted to AF-drug 4g, 2+ in the presence of H2O2 (Figure S29, S30) that is accompanied by the strong (up to 50-fold) fluorescence increase (Figure S39). We found that 4g is accumulated and activated in A2780 cells leading to formation of fluorescent products (most probably 4g, 2+) that could be monitored by flow cytometry (Figure S40, Table S9). By using fluorescence microscopy we confirmed that the fluorescent product derived from 4g is accumulated in ER (Figure 2C–K). This is evident from the efficient overlap (Pearson’s R value = 0.85, Figure S41, SI) of the signal of the fluorescent product 4g, 2+ derived from 4g (green color) and the signal of ER-specific stain ERTrEd (red color, Figure 2K). It should be mentioned that previously reported fluorogenic versions of LY-targeting 2[13] and Mit-targeting 4[14] are substantially less active than their unlabeled counterparts. These compounds are suitable for the study of the mechanism of prodrug action, but not as therapeutic agents. In contrast, 4g is the first truly teranostic AF-based prodrug.

To evaluate the cancer cell specificity of the ER-targeting AF-prodrugs, we conducted two complementary experiments. For the first one we selected a pair of genetically related primary cancer (chronic lymphocytic leukemia: CLL) and normal cells (mononuclear cells: MNCs). MNCs is a mixture of cells containing primary B cells, which are parent for CLL cells. We observed that 4a is significantly more toxic towards CLL cells (IC50 = 5.6 ± 0.7 μM) than normal MNCs (IC50 = 18.3 ± 7.5 μM, p < 0.05) (Figure S42, SI). Furthermore, by using fluorescence microscopy we confirmed that 4g (the fluorogenic analogue of 4a) is activated only in cancer (A2780), but not in normal (SBLF9 fibroblasts) cells (Figure S43, SI). We selected the SBLF9 cells as representative normal cells, since they are adherent and, therefore, better suitable for the fluorescence microscopy than the non-adherent MNCs.

Finally, we evaluated the activation of 4g in vivo in C57/BL6N mice carrying NK/Ly and treated with 4g for 3 days. B) Increase of the mean fluorescence (λex = 340–380 nm; λem = 435–485 nm) of ascites isolated from the C57/BL6N mice treated with 4g for 0, 3 and 3 days: circles: individual data, horizontal bars: means of the individual data. Student’s t test: ***p < 0.001; **p < 0.01; *p < 0.05; λem = 415–465 nm (D) and λem = 530–570 nm; λex = 570–650 nm (E). F) Overlay of images shown in (D) (green color) and (E) (red color). G–J Controls; the same as (C–F) for untreated ascites.

day for 3 days. Ascite probes were taken 3 h and 3 days after the beginning of the treatment. The cell suspensions appeared yellow indicating the prodrug uptake (Figure 3A). Furthermore, the fluorescence of ascites (λex = 360/20 nm, λem = 460/ 25 nm) was quantified by using a fluorescence plate reader (SI). We observed the significant (p < 0.01 for 3 h incubation and p < 0.001 for 3 days incubation) time-dependent fluorescence increase in the treated animals compared to the non-treated ones (Figure 3B) that is in agreement with the activation of 4g in vivo. These results were confirmed by imaging of the live ascites by using vital fluorescence microscopy (3 days incubation, Figure 1C–J). In particular, we imaged the cells at two settings (Ch1 and Ch2), which allowed detecting the products of 4g activation (Ch1: λex = 290–410 nm; λem = 415–465 nm (D) and λem = 530–570 nm; λex = 570–650 nm (E)). We confirmed that 4g is accumulated and activated in the ER of the ascites in vivo. As expected, no signal in the Ch1 was observed in the ascites isolated from the control (untreated) group. These data are in agreement with our in vitro studies (Figure 2C–K).

In summary, we successfully prepared cholic acid-conjugated AF-prodrug 4a as well as its fluorogenic version 4g, which is the first reported teranostic AF-prodrug. We demonstrated fast (≤ 1 h incubation) accumulation and activation of these prodrugs in the ER of cancer cells that leads to the significant ER stress (upregulation of CHOP mRNA) and the production of both mitochondrial and total ROS. We have confirmed the excellent cancer cell specificity of prodrugs 4a/4g and demonstrated that 4g is efficiently activated in vivo in the NK/Ly murine model. The prodrugs 4a/4g described in this paper and the previously reported LY-targeting 2[13] exhibit comparable anticanic effects. However, due to different mechanisms of action, these drugs are...
complementary to each other. We assume that 4a/4g may find applications in cases when 2 is not suitable. Furthermore, the simultaneous use of these produgs would be an interesting option due to the possible synergistic effects between them.

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Conflict of interest

The authors declare no conflict of interest.

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