A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition Chemie

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Title: Lysosome-targeting amplifiers of reactive oxygen species as anticancer prodrugs

Authors: Andriy Mokhir, Steffen Daum, Viktor Reshetnikov, Miroslav Sisa, Tetyana Dymych, Maxim D Lootski, Rostyslav Bilyy, Evgenia Bila, Christina Janko, Christoph Alexiou, Martin Herrmann, and Leopold Sellner

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201706585
Angew. Chem. 10.1002/ange.201706585

Link to VoR: http://dx.doi.org/10.1002/anie.201706585
http://dx.doi.org/10.1002/ange.201706585

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Lysosome-targeting amplifiers of reactive oxygen species as anticancer prodrugs

Steffen Daum,[a] Viktor Reshetnikov,[a] Miroslav Sisa,[a,b] Tetyana Dumych,[c] Maxim D. Lootsik,[c] Rostyslav Bilyy,[c] Evgenia Bila,[d] Christina Janko,[e] Christoph Alexiou,[e] Martin Herrmann,[f] Leopold Sellner[g] and Andriy Mokhir*[a]

Abstract: Cancer cells produce elevated amounts of reactive oxygen species that has been used to design cancer specific prodrugs. Their activation relies on at least a bimolecular process, where a prodrug reacts with ROS. However, at low μM concentrations of the prodrugs and ROS the activation is usually inefficient. Herein we suggested and validated a potentially general approach for solving this intrinsic problem of ROS-dependent prodrugs. In particular, known 4-(N-ferrocenyl-N-benzylaminocarbonyloxymethyl)phenylboronic acid pinacol ester was converted to its lysosome specific analogue. Since lysosomes contain the higher concentration of active ROS than cytoplasm, activation of the latter prodrug was facilitated with respect to the parent compound. In particular, it was found to exhibit high anticancer activity in a variety of cancer cell lines (IC_{50} 3.5-7.2 μM) and *in vivo* (40 mg/kg, NK/Ly murine model), but remained weakly toxic towards non-malignant cells (IC_{50} 15-30 μM).

The mode of action of the majority of chemotherapeutic drugs clinically approved for the treatment of hematologic cancers including alkylating agents, antimetabolites, anthracyclines and alkaloids relies on targeting quickly growing cells. However, apart from cancer cells, some types of non-malignant cells also exhibit such a property. Therefore, though great progress has been achieved in this field over the last decades,[1] the cancer-cell specificity of chemotherapeutics is still low and side effects are dose-limiting. This problem can be approached by using targeted drugs including either monoclonal antibodies (e.g. alemtuzumab, ofatumumab)[2] or low molecular weight inhibitors of enzymes, which are either cancer specific or overexpressed in cancer cells, e.g. bcr-abl tyrosine kinase and Bruton's tyrosine kinase (Btk).[3] However, cancers, which rely on a single receptor or enzyme, are rare. Moreover, blood cancers are often genetically heterogeneous and can evade the action of molecular targeted drugs due to their intrinsic genetic instability leading to quick mutations of the target biomolecules.[4]

More robust targeting can be potentially achieved by prodrug activation at the cancer specific microenvironment. For example, the majority of cancer cells overproduce reactive oxygen species (ROS),[5] whereas in non-malignant cells their concentration is extremely low (0.001-0.7 μM).[6] Since elevated ROS seems to be a general feature of the cancer phenotype, prodrugs activated by ROS are potentially applicable for the treatment of many different cancer types.[7] Several such prodrugs were reported. In particular, Jaouen and co-workers have developed ferrocene analogues of tamoxifen called ferrocifens and their analogues, which are converted under oxidative conditions into electrophilic products able to deactivate thioredoxin reductase.[8] Jacob and co-workers have reported on organochalcogenide-based prodrugs, which catalyze oxidation of glutathione (GSH) to GSSG by H_2O_2.[9] The group of Peng have introduced pro-alkylating agents, which react with H_2O_2 resulting in formation of electrophilic DNA cross-linking agents.[10] Finally, our group has developed N-alkylaminoferrocene-based prodrugs, which are converted to an ROS amplifier (electron rich ferrocene) and an alkylating agent (quinone methide) under cancer specific conditions. Both these reagents act synergistically by increasing oxidative stress in cancer cells thereby causing their death.[11]

The intracellular concentration of H_2O_2 (the most stable ROS, $[H_2O_2]_{in}$) in cancer cells was estimated to be in the low μM range: e,g, for Jurkat T-cells it is $\leq$7 μM,[5a] whereas concentrations of more reactive ROS (e.g. $O_2^{\bullet-}$, HO•) are expected to be even lower. The activation of the ROS-dependent prodrugs occurs in at least a bimolecular process (a drug reacts with an oxidant), whose rate is strongly dependent upon concentrations of the reagents: reaction rate (v)= constant(k)*[prodrug][ROS]. Providing the prodrug is used at concentrations below 10 μM, the product [prodrug][ROS] is expected to be $<10^{-10}$ mol/L that means that only very quick reactions (large k) are suitable for the efficient oxidative activation of prodrugs. It is an important intrinsic limitation of the ROS-dependent prodrugs, since such reactions are usually rare and not well compatible with aqueous buffers. Arguably, it can be one of the reasons why not a single ROS-responsive anticancer prodrug has been clinically approved until now despite their great potential.

Herein we suggest a potentially general solution of this problem using N-alkylaminoferrocene-based prodrugs as an example. In particular, we converted a known prodrug **1**[11e] to its analogues (prodrugs **2** and **5**, Figure 1), which target lysosomes (LY's) due to the presence of an alkylated piperidine fragment. The latter moiety is protonated in the acidic environment of LY leading to

[a] S. Daum, MSc; Dr.; V. Reshetnikov, MSc; M. Sisa; A. Mokhir, Prof. Dr. Friedrich-Alexander-University of Erlangen-Nürnberg, Department of Chemistry and Pharmacy, Organic Chemistry Chair II, Henkestr. 42, 91054 Erlangen, Germany
E-mail: Andriy.Mokhir@fau.de
[b] M. Sisa, Dr., Institute of Experimental Botany AS CR, Prague, Czech Republik
[c] R. Bilyy, Prof. Dr.; T. Dumych, PhD; M.D. Lootsik, Prof. Dr.; Danylo Halytsky Lviv National Medical University, Pekarska str. 69, 79010 Lviv, Ukraine
[d] E.Bila, Dr.; Department of Organic Chemistry, Ivan Franko Lviv National University, Kyrylo & Mefodiy Str. 6, 79005, Lviv, Ukraine
[e] C. Janko, Dr.; C. Alexiou, Prof. Dr. med., Department of Otorhinolaryngology, Head and Neck Surgery, Section of Experimental Oncology and Nanomedicine (SEON), University Hospital Erlangen, Glückstraße 10a, 91054 Erlangen, Germany
[f] M. Herrmann, Prof. Dr. Dr.; Department of Internal Medicine 3 - Rheumatology and Immunology, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91054 Erlangen, Germany
[g] L. Sellner, Dr.; Department of Medicine V, University Hospital Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany

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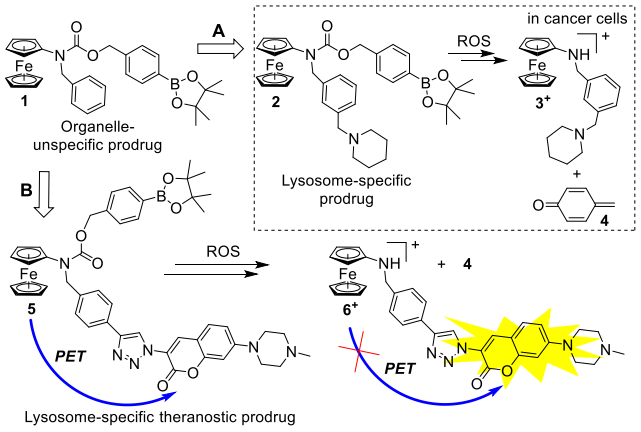

Figure 1. Structures of aminoferrocene-based prodrugs **1**, **2** and **5** as well as the mechanism of their activation in cancer cells (insert). Paths **A** and **B**: conversions of unspecific prodrug **1** to their lysosome-specific analogues **2** (non-fluorogenic) and **5** (fluorogenic).

trapping of the hybrid. LY was selected as a target, since H_2O_2 produced by a cancer cell freely enters its LY's via diffusion. Since, in contrast to cytoplasm, LY's do not contain H_2O_2-degrading enzymes,[12] H_2O_2 can get accumulated.[13] Additionally, these organelles contain large amounts of loosely bound iron ions, which catalyze conversion of H_2O_2 into highly toxic HO•.[14] Among all ROS, the latter one is the most efficient oxidizer of N-alkylaminoferrocene-based prodrugs.[11e] Thus, the prodrug activation in this case will be facilitated by three factors: prodrug accumulation in LY's, increased concentration of ROS in these organelles and higher fraction of the most active ROS - HO•.

Prodrug **2** was prepared as described in the supporting information (*SI*). The LY-targeting was achieved by addition of a basic aliphatic tertiary amine residue (piperidine) to the parent structure **1**. Such residues act as LY-specific carriers, since they are protonated in the acidic environment of LY leading to cargo accumulation within this organelle.[15]

First, we investigated basic properties of prodrug **2** and compared them to those of the previously reported prodrug **1**.[11e] In particular, we found that lipophilicities of **1** and **2** are similar: logP= 4.5 ± 0.3 (for **1**); 4.7 ± 0.1 (for **2**). In agreement with these data, they permeate the cellular membrane of Burkitt lymphoma BL-2 cells with the same efficiency (*SI*). By using ESI mass spectrometry we confirmed that prodrug **2** is converted to its predicted products in the presence of H_2O_2 (Figures 1, S4-S6): B-C cleavage product I (*m/z* 539.2000, $[M+H]^+$) and N-alkylaminoferrocene II (*m/z* 388.1594, $[M]^+$). Additionally, minor peaks corresponding to the product of decomposition of the ferrocene unit in II (*m/z* 267.1858, $[M+H]^+$) and the product of mono-alkylation of II with quinone methide **4** (*m/z* 494.2018, $[M]^+$) were detected (*SI*). Thus, the mechanism of H_2O_2-induced activation of **2** remains the same as that of **1**.[11] In agreement with the latter statement, we observed that prodrug **2** facilitates conversion of H_2O_2 to HO•. The latter reaction was followed by HO•-mediated oxidation of non-fluorescent 2',7'-dichlorofluorescin (DCFH) to fluorescent 2',7'-dichlorofluorescein (DCF). Interestingly, we found that the catalytic efficiency of the new prodrug is ~4-fold higher than that of the parent prodrug **1**: $(dF/dt)_0$= 7.81 s^{-1} for **2**, 1.97 s^{-1} for **1** and 0.02 s^{-1} for a background reaction (Figure 2A). Previously, we confirmed that the low activity of **1** could be attributed to its aggregation in aqueous solution that inhibits its interaction with H_2O_2.[16] Here we found that in contrast to **1**, prodrug **2** has lower propensity towards aggregation (*SI*) that explains its higher reactivity towards H_2O_2.

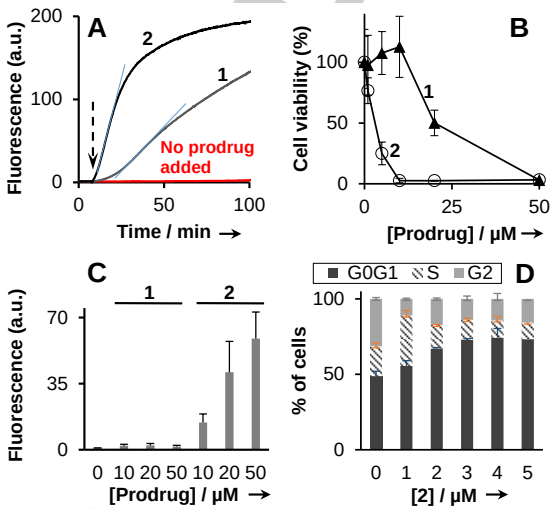

Figure 2. A: Increase of the fluorescence intensity (λ_{ex} = 501 nm, λ_{em} = 531 nm) upon oxidation of 2',7'-dichlorofluorescin (DCFH, 9.9 μM) by H_2O_2 (9.9 mM) either in the presence of prodrugs **1** and **2** (both 49.5 μM) or in their absence. The time point of the prodrug addition is indicated with a black dashed arrow. After the initial lagging period, the oxidation reactions reach their maximum rates. Linear fits of these regions are indicated with blue lines. Other conditions are given in the *SI*. **B**: Effects of prodrugs **1** (filled triangles) and **2** (open circles) on the viability of BL-2 cells. **C**: Increase of the mean fluorescence (λ_{ex} = 488 nm, λ_{em} = 530 nm, monitored by flow cytometry) of 5-(6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCFH-DA)-loaded BL-2 cells incubated with prodrugs **1** and **2** for 2 h. **D**: Effect of prodrug **2** (incubation for 24 h) on cell cycle of BL-2 cells.

Encouraged by these data, we studied toxicity of the new prodrug **2** towards representative human blood cancer cell lines derived from B- (BL-2) and T-cells (Jurkat) (Figure 2B, Table S1). In both cases we observed that the piperidine derivative **2** is substantially more toxic than the parent **1**: 3.5 ± 0.9 *vs* 26 ± 5 (BL-2, $p< 0.001$) and 7.2 ± 0.1 *vs* 44 ± 2 μM (Jurkat, $p< 0.001$). In agreement with these data prodrug **2** induces 7-35 fold stronger oxidative stress in BL-2 cells than its analogue **1** (Figure 2C). The induction of the oxidative stress in BL-2 cells by the prodrugs is accompanied by initial upregulation of the intracellular antioxidant glutathione ($[GSH]_{in}$, $p<0.01$) as measured by staining with GSH-specific dye monobromobimane (MBB, Figure S12). Furthermore, prodrug **2** causes BL-2 cell cycle arrest in G0/G1 phase: 73 ± 3 % at [**2**]= 3-5 μM *vs* 48 ± 3 % in the absence of any prodrug (Figure 1D). We also observed the similar effect for prodrug **1** but at the substantially higher concentrations: G0/G1 59-66 % at [**1**]= 25-30 μM (Figure S13). Incubation of Jurkat cells with the prodrugs causes similar changes in $[GSH]_{in}$ (Figure S21), namely upregulation of GSH for prodrug **2** ≤ 12.5 μM and oxidation of GSH to GSSG for prodrug 2 ≥ 25 μM (t= 24h). For the latter cell line we studied the mechanism of prodrug-induced cell death (Figure S20). In particular, we found that at the low [**2**] the cells

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are killed via both apoptosis and necrosis mechanisms, whereas in the presence of the high [**2**] necrosis dominates. The cell-kill could be partially rescued by the addition of N-acetylcystein (NAC), which indicates that ROS plays is an important determinant of the mode of action of prodrugs **1** and **2**. Based on these data, we could conclude that the introduction of a piperidine residue to the structure of **1** dramatically improves the activity of the prodrug both in cell free settings and in cell lines. Importantly, the toxicity of the resulting compound against normal, non-cancer human dermal fibroblast adult (HDFa) cells remains low (IC_{50}= 30 ± 1 μM) relative to that for BL-2 ($p<0.001$) and Jurkat cells ($p<0.001$) (Table S1). Moreover, the new prodrug is highly active against primary chronic lymphocytic leukemia (CLL) cells (IC_{50}= 2.0 ± 1.1 μM), but remains only weakly toxic against normal mononuclear cells (MNC's, IC_{50}= 15 ± 7 μM, $p<0.01$), which contain a fraction of B cells. The latter is an excellent drug toxicity model, since both CLL- and B-cells are genetically related to each other.[11b]

Based on the initial design prodrug **2** was supposed to target LY's of cancer cells. To evaluate whether this is the case, we labeled LY's in BL-2 cells with acridine orange (AO). AO exhibits orange fluorescence when accumulated in LY's. In contrast, it emits in the green spectral region when bound to RNAs.[17] We detected the LY-specific emission of the cells (F_{LY}) by using flow cytometry (λ_{ex} = 488 nm, λ_{em} = 690 nm, Figure S14). In the untreated, AO-loaded cells mean F_{LY} was found to be high: 176 ± 5 arbitrary units (a.u.). The treatment of the cells with prodrug **1** led to only slight decrease of mean F_{LY} (111 ± 6 a.u., $p<0.001$). In contrast, the cells treated with prodrug **2** were practically non-fluorescent (mean F_{LY} = 19 ± 1, $p<0.001$). These data indicate that prodrug **2** causes LY-disruption in BL-2 cells that probably is the main cause of prodrug **2**-induced cell death. We also reproduced this effect in human prostate DU-145 cells (Figure S15). The latter cells were selected since they are adherent and, therefore, can be well studied using fluorescent microscopy. Moreover, DU-145 cells are known to generate large amounts of ROS[18] and are, therefore, responsive to aminoferrocene-based prodrugs.[11c] In particular, we observed that similarly to BL-2 and Jurkat cells, prodrug **2** was more toxic towards DU-145 cells (IC_{50}= 6.5 ± 0.1) than prodrug **1** (IC_{50}= 24.5 ± 0.1). By using fluorescence microscopy, we found that untreated, AO-loaded DU-145 cells contain yellow dots on the green background, which could be identified as LY's (Figure 3, image A). The same pattern was observed in the cells treated with prodrug **1** (image B). In contrast, the cells treated with prodrug **2** lack yellow dots that confirms the drug-induced LY-disruption in this case (image C). Accumulation of prodrug **2** by LY's could not be studied directly by the previously reported assay based on the analysis of boronic acid released in cells upon activation of the prodrugs, due to its insufficient sensitivity.[11] Therefore, we introduced an alternative fluorescence-based assay: for that purpose, an analogue of prodrug **2** (prodrug **5**) containing a coumarin dye was synthesized (*SI*). In the intact state prodrug **5** is practically not fluorescent due to the efficient photoinduced energy transfer (PET) from the ferrocene moiety to the dye (Figures 1, S3). However, upon its treatment with H_2O_2 it is activated with formation of ferrocenium derivative **6**$^+$ and quinone methide **4** as outlined in Figure 1 and experimentally confirmed by ESI mass spectrometry (Figures S7-S9). Since, in contrast to the starting prodrug, product **6**$^+$ does not contain any electron donor, PET is not possible and this compound is fluorescent (Figure S3). Both prodrugs **2** and **5** were found to have similar lipophilicities and cell membrane permeabilities (*SI*). Furthermore, we observed that prodrug **5** is toxic towards BL-2 cells (IC_{50}= 27 ± 3 μM). The lower toxicity of **5** with respect to **2** ($p< 0.001$) was explained by the decreased ROS-generating ability of **5** (Figure S11), which is caused probably by coumarin-induced ROS quenching.

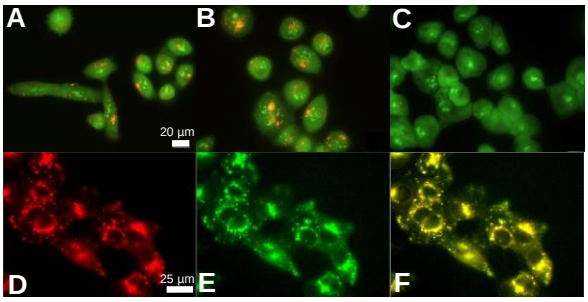

Figure 3. A-B: Acridine orange (AO)-loaded DU-145 cells; no prodrug added (**A**); in the presence of podrug **1** (**B**) or prodrug **2** (**C**). **D-E**: DU-145 cells treated with Lysotracker Red (LTR) and prodrug **5**; red channel is LTR specific (**D**), green channel is specific for products released from **5** in cells (**E**); image **F** is an overlap of **D** and **E**.

Next, we co-incubated prodrug **5** and Lysotracker Red (LTR: a LY-specific dye) with DU-145 cells and imaged the cells with fluorescence microscopy. The LTR dye was detected by using excitation / emission combination of 538-562 / 570-640 nm (red color in Figure 3D), whereas the activated **5** was detected by using excitation / emission combination of 335-383 / 420-470 nm (green color, E). Overlap of two images appears yellow (F) that indicates that both dyes are fully co-localized. This experiment confirms unambiguously that prodrug **5** is accumulated in LY's and gets activated in these organelles. The LY-disruption was not observed at the concentrations of the prodrug used in this experiment. These data were confirmed by flow cytometry (Figure S17).

Finally, antitumor activity of prodrug **2** was evaluated in murine Nemeth-Kellner lymphoma (Nk/Ly) model.[19] Two preliminary experiments including 6 and 13 black C57/BL6N mice, in which the dose of **2** was optimized, and one final experiment with 14 mice (m~25 g) were performed. In particular, the mice were inoculated subcutaneously with $7.5*10^5$ NK/Ly cells (day 0), that initiated the growth of tumor according to lymphosarcoma type. Then either a carrier (DMSO, 30 μL, control group, N=7) or prodrug **2** dissolved in the carrier (30 μL, dose 40 mg/kg, test group, N=7) were injected intraperitoneally (*i.p.*) on days 1, 3, 5, 7, 9, 11, 13, 15. The increase of weight of animals, which correlates with tumor progression, was monitored. We observed that prodrug **2** inhibits tumor growth significantly (Figure 4A). On day 19 the animals were sacrificed, tumors excised, weighed, fixed and undergone histological analysis. In all animals except of one in the test group the tumor weight was substantially reduced with respect to those from the control group (Figure 4B). Furthermore, histological analysis of lymphosarcoma from the group treated with DMSO alone exhibited prominent signs of neoangiogenesis (marked V) and abundant mitoses (Figure C),

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whereas the presence of the large number dying cells (both apoptotic and necrotic) were observed in tumors from the group treated with prodrug **2** (Figure 4D).

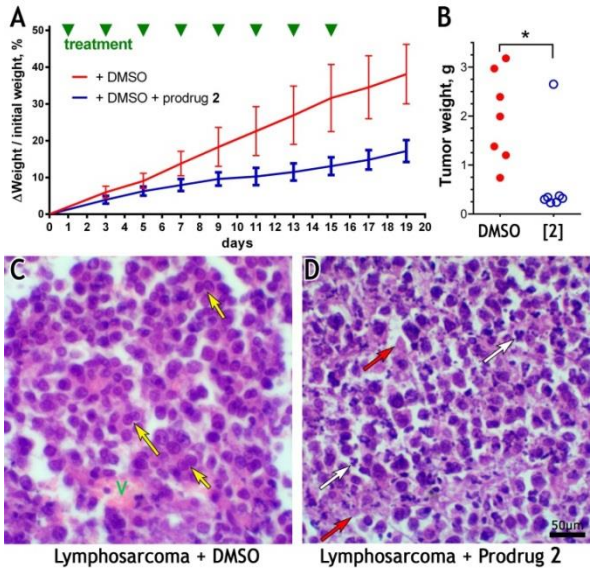

Figure 4. **A**: Animal growth curves. **B**: Tumor weight. **C**: Histology of a slice of the non-treated tumor. **D**: Histology of a slice of the treated tumor.

In summary, we confirmed that the aminoferrocene-based prodrugs, which carry a dialkylamino-residue (prodrug **2** and its fluorogenic analogue **5**), target lysosomes in cancer cells. Due to the presence of excess ROS in these organelles, the activation of these prodrugs is significantly facilitated with respect to the best previously reported aminoferrocene-based prodrug **1**. Prodrug **2** kills cancer cells via both apoptosis and necrosis at low prodrug concentrations and mainly necrosis at higher concentrations. The cytotoxicity mechanism includes lysosome disruption, [ROS] increase, cell cycle arrest in G0/G1 phase and [GSH] increase. Prodrug **2** was found to be highly active not only towards cancer cell lines (BL-2, Jurkat, DU-145), but also towards primary cells (CLL cells) and *in vivo* (NK/Ly mouse model). Importantly, its activity towards non-malignant cells (HDFa, NMC's) was substantially reduced. The approach reported here for improvement of the activity of aminoferrocene-based prodrugs can be potentially applied towards other ROS-dependent prodrugs. Furthermore, it will facilitate the progress in the field of "smart" redox regulating agents and help to advance these to clinical trials.

Experimental Section

Synthesis of new prodrugs, description of assays and additional experimental data are provided in the supporting information (*SI*).

Acknowledgements

AM thanks German Research Council (DFG, MO1418 / 7-1) for funding this project. CA and CJ were supported by the DFG through the Cluster of Excellence Engineering of Advanced Materials (EAM).

Keywords: Aminoferrocene • Prodrug • Lysosome • Cancer • Reactive Oxygen Species

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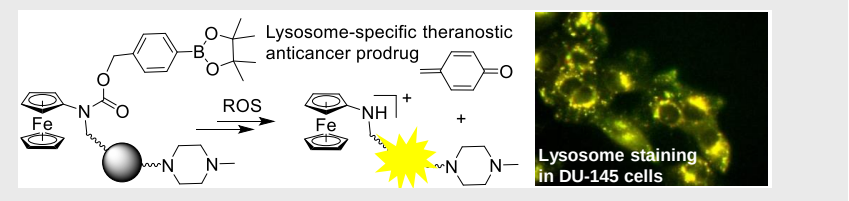

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*Steffen Daum, Viktor Reshetnikov, Miroslav Sisa, Tetyana Dymych, Maxim D. Lootsik, Rostyslav Bilyy, Evgenia Bila, Christina Janko, Christoph Alexiou, Martin Herrmann, Leopold Sellner and Andriy Mokhir**

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Lysosome-targeting amplifiers of reactive oxygen species as anticancer prodrugs

Accepted Manuscript

WILEY-VCH

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