Anticancer Aminoferrocene Derivatives Inducing Production of Mitochondrial Reactive Oxygen Species

Hülya Gizem Özkan,[a] Vanrajsinh Thakor,[a] Hong-Gui Xu,[a] Galyna Bila,[b] Rostyslav Bilyy,[b] Daria Bida,[a] Martin Böttcher,[c] Dimitrios Mougiakakos,[c] Rainer Tietze,[d] and Andriy Mokhir*[a]

Abstract: Elevated levels of reactive oxygen species (ROS) and deficient mitochondria are two weak points of cancer cells. Their simultaneous targeting is a valid therapeutic strategy to design highly potent anticancer drugs. The remaining challenge is to limit the drug effects to cancer cells without affecting normal ones. We have previously developed three aminoferrocene (AF)-based derivatives, which are activated in the presence of elevated levels of ROS present in cancer cells with formation of electron-rich compounds able to generate ROS and reduce mitochondrial membrane potential (MMP). All of them exhibit important drawbacks including either low efficacy or high unspecific toxicity that prevents their application in vivo up to date. Herein we describe unusual AF-derivatives lacking these drawbacks. These compounds act via an alternative mechanism: they are chemically stable in the presence of ROS, generate mitochondrial ROS in cancer cells, but not normal cells and exhibit anticancer effect in vivo.

Introduction

A common consequence of transformation of normal to cancer cells is altered homeostasis of reactive oxygen species (ROS). The transformed cells steadily produce ROS leading to their sensitive to exogenous ROS than normal cells.[1] As a consequence, drugs producing ROS can exhibit anticancer effects.[2] Since ROS are highly cytotoxic, the challenge in the therapeutic application of such drugs is to achieve cancer cell specificity to avoid expected detrimental effects on normal cells and tissues.

To address this issue, we have introduced cancer specific aminoferrocene (AF)-based ROS amplifiers (type A agents, Figure 1A, the activation is triggered by B–C oxidation).[3] Other known drugs/agents activated in the presence of ROS and amplifying ROS in cells include procarbazine (HN-NH oxidation),[3] hydroxyferrocifenes (C–H oxidation)[4] and organochalcogenides (RXR oxidation, where X=Se or Te).[3]

Activation of the AF-agents starts with spontaneous hydrolysis of the boronic acid pinacol ester moiety (agents 1a–6a, Figure 1A) with formation of boronic acid derivatives (drugs 1b–6b). These further react with ROS, triggering the reaction sequence that leads to formation of AF Fc–NH–R'. The activation occurs only in ROS-rich (cancer) cells. Redox potentials of Fc–NH–R' products are shifted ~300 to ~400 mV with respect to the parent agents. These electron rich catalysts are capable of donating an electron to hydrogen peroxide (H₂O₂) thereby transforming this less reactive ROS to highly reactive and toxic hydroxyl radicals (HO·).[5] The latter species can be detected in cytoplasm by using 5(6)-chloromethyl-2',7'- dichlorodihydro-fluorescein diacetate (CM-DCFH-DA). Since the intracellular localization of CM-DCFH-DA is not biased, it senses ROS in the whole cell. In this paper we call the CM-DCFH-DA-sensitive intracellular pool of ROS total ROS or tROS. Both previously developed AF derivatives 2a[6] and 3a[6] exhibiting anticancer effects in vivo, were found to strongly induce tROS generation. In contrast, they either do not or weakly affect mitochondrial ROS (mROS), detected by using MitoSOX™ probe.

Apart from the elevated ROS, mitochondria functions in transformed cells are partially impaired. Therefore, glycolysis is switched from oxidative phosphorylation, which is typical for normal cells, to anaerobic metabolism.[6] However, these cells still rely on remaining mitochondrial functions, including, for example, triphosphate and nucleotide synthesis.[8] Correspond-
The observed problems with mROS-generating AF drugs are primarily due to two weak points of cancer cells: they support stress signaling and induce mutations in mitochondrial and genomic DNAs thereby promoting neoplastic transformation.[13] Therefore, the cancer vs. normal cell specificity for mROS-generating agents should be especially high to be able to achieve useful therapeutic effects.

We have serendipitously discovered that AF derivatives, in which a ROS-sensitive moiety is replaced with a ROS-resistant fragment (R" = F, type B agents, Figure 1B), generate mROS in cancer cells, whereas normal cells remain unaffected. In this paper, we report on detailed studies of these new agents as well as their close analogues including their synthesis, characterization and basic properties. We studied the mechanism of action of type B agents and evaluated their anticancer efficacies in vitro (on selected cancer cell lines and primary cells) and in vivo (murine Nemeth-Kellner lymphoma, NK/Ly).

Results and Discussion

Reference compounds 1a–5a were prepared as previously reported.[9,10,11] Compounds 1c–1e, 2c, 2d, 3d, their brominated 3d_Br (Figure 1B) and fluorogenic 6d, 7d analogues as well as control 6a were prepared as described in detail in the Supporting Information. All new compounds were >95% pure according to C, H, N analysis. Their solubility in phosphate-buffered saline (PBS, pH 7.4) exceeded 25 μM and in Roswell Park Memorial Institute (RPMI) medium containing 5% of fetal bovine serum (FBS) - 100 μM (Table S1, Supporting Information).

Preliminary studies

Type A agents (e.g. 1a–6a) lose the pinacol fragment in aqueous neutral solutions within ~2 h forming boronic acids (1b–6b). As previously reported,[14] the latter species are responsible for the anticancer activity of these agents. In this work, we used ROS-resistant N,N-dimethylaminocarbonyl (A) analogues as negative controls since the polarity of the A fragment matches that of the boronic acid fragment. The latter is confirmed by similar n-octanol/water partition coefficients (logP's) of the following pairs of agents and controls: logP(1b) = 2.76 ± 0.23 vs. logP(1c) = 3.36 ± 0.11 and logP(2b) = 3.35 ± 0.25 vs. logP(2c) = 3.96 ± 0.15 (Table 1). Based on these data, it is expected that passive cellular uptake of the agents and the controls is comparable to each other. Therefore, differences in their activity should not be related to different uptake efficacies. In contrast to 1a and 2a, negative controls 1c (E10 = −70 mV vs. FcH) and 2c (E10 = −10 mV vs. FcH) cannot be transformed via route 1 to the electron rich Fe–NH–R" products (E10 = −300 to −400 mV vs. FcH, Figure 1A). Correspondingly, they (a) are poor catalysts for ROS generation in cell free settings with the efficacies slightly higher than those of ferrocene (FcH, Table 1), (b) induce production of less tROS in representative human cancer ovarian (A2780) cells and (c) are less toxic to A2780 cells than the corresponding drugs 1a and 2a (Table 1). In spite of this expected trend, we were puzzled by the fact that both 1c and...
Table 1. Comparison of selected properties of type A and B agents.

| Agent | LogP[a] | E1/2[b] | ROS releasing efficacy[c] | IC50 [μM][d] | Cells |
|-------|---------|---------|--------------------------|--------------|-------|
| 1a    | 5.00 ± 0.30 | -70 | 57 ± 7 | 23.9 ± 2.7 | A2780 cells |
| 1b    | 2.76 ± 0.23 | - | - | - | - |
| 1c    | 3.36 ± 0.11 | -70 | 6 ± 1 | 46.1 ± 8.5 | - |
| 1d    | 4.17 ± 0.27 | -76 | 7 ± 1 | 15.0 ± 2.4 | - |
| 1e    | 1.90 ± 0.04 | -70 | 8 ± 1 | > 50 | - |
| 2a    | 5.51 ± 0.17 | -10 | 54 ± 7 | 14.2 ± 2.1 | - |
| 2b    | 3.35 ± 0.25 | - | - | - | - |
| 2c    | 3.96 ± 0.15 | -10 | 2.5 ± 0.7 | 37.1 ± 6.2 | - |
| 2d    | 4.72 ± 0.15 | -15 | 1.1 ± 0.3 | 16.7 ± 2.5 | - |
| 3a    | > 6.26 | -40 | 54 ± 6 | 5.6 ± 1.7 | - |
| 3b    | 6.09 ± 0.03 | - | - | - | - |
| 3c    | 5.79 ± 0.03 | -35 | 5 ± 1 | - | - |
| 3d    | 6.05 ± 0.07 | -35 | 2.7 ± 0.8 | 7.0 ± 1.3 | - |
| 3d_Br | 6.22 ± 0.07 | 116 | 4.3 ± 0.9 | 6.2 ± 0.1 | - |
| FcH   | 3.40 | 0 | 0.4 ± 0.2 | > 50 | > 50 |

[a] LogP: n-octanol/water partition coefficients. [b] E1/2: redox potentials versus ferrocene/ferrocenium redox couple (FcH/FcH+) in mV. [c] ROS releasing efficacy is expressed as the maximal increase of the fluorescence intensity of 2',7'-dichlorofluorescin in the presence drugs/controls ((dF/dt)μM, μM, Fig- ure 2). [d] IC50: a concentration, at which half of the cells remain viable; A2780: human ovarian cancer cells; BL-2: human Burkitt's lymphoma cells.

and 2c increase the level of tROS in A2780 cells to some degree (1c by 2.2 ± 0.6; 2c by 6.1 ± 0.7 fold as compared to the effect of the carrier only (DMSO)) and exhibit the moderate anticancer activity. In contrast, ferrocene, which has similar to 1c and 2c redox potential (Table 1), is not toxic under these conditions. The mechanism of the ROS production by 1c and 2c is not related to that of the previously known AF agents. In the following sections of this paper we will use term “type B” for the new agents originally derived from negative controls 1c and 2c with R’ = A, F and CA (Figure 1).

Optimization of the anticancer activity of type B agents

With the goal to understand the effect of lipophilicity on the anticancer activity of type B agents, we prepared two analogues of 1c containing fluorine (1d) and a carboxylic acid residue (1e) in place of the “A” fragment. 1d is more (logP = 4.17 ± 0.27) and 1e (logP = 1.90 ± 0.04) – less lipophilic than 1c. Redox potentials as well as ROS releasing ability in cell free settings of all these compounds are practically identical (Table 1). We found that the cytotoxicity towards A2780 cells in this small series correlates with lipophilicity (Table 1). To further optimize most active 1d, we introduced at its carbamate nitrogen either propargyl (P) or 3-(N-piperidinomethyl)benzyl (M) fragments to obtain 2d and 3d, correspondingly. Though 2d is slightly more lipophilic than 1d, it does not lead to the higher cytotoxicity towards A2780 cells. In contrast, substantially more lipophilic 3d (3d: logP = 6.05 ± 0.07) exhibits the highest activity among all new drugs reported in this paper: IC50 (3d) = 7.0 ± 1.3 μM (Table 1). The effect is not only restricted to one cell line. In particular, 3d is active against Burkitt’s lymphoma BL-2 cells (IC50 (3d) = 3.3 ± 0.3 μM, Table 1) as well as primary chronic lymphocytic leukemia (CLL) cells (IC50 (3d) = 4.8 ± 2.0 μM, Figure 2).

The efficacy of 3d against all studied cells (A2780, BL-2 and CLL cells) was found to be the same as that of the corresponding type A agent 3a (Table 1, Figure 2, Student’s t test).

Figure 2. Effect of agents 3a (inset A) and 3d (inset B) on viability of primary cancer and normal cells (CLL, B and T cells). C: Effects of 3d and 3d < T > Br on viability of CLL cells. Student’s t test: * - p < 0.05, ns – non-significant, p> = 0.05.
A mechanism of uptake and anticancer activity of agent 3d

First, we investigated the uptake of 3d and known agent 3a by A2780 cells by monitoring the increase of iron amount ([Iron]) in the cells treated with the drugs (Figure 3A). To determine the mechanism of this process, we conducted the experiment at 37 and 4 °C (Figure 3A). At 37 °C the uptake of both agents was the same and strong, whereas at 4 °C it was significantly inhibited for 3a, but not affected for 3d. These data indicate different uptake mechanisms of the agents. For 3a it is at least partially energy dependent (active or facilitated) and for 3d – energy independent (passive).

Furthermore, we found that analogously to 3a agent 3d induces the statistically significant increase of iROS in A2780 cells. However, the effect of 3d is substantially weaker than that of 3a (p < 0.05, Student’s t test, Figure 3B). Interestingly, the low iROS generated in 3d-treated cells is still important for the anticancer activity of the agent. This is confirmed by the concentration dependent attenuation of cytotoxicity of 3d in the presence of the ROS scavenger N-acetyl cysteine (NAC, Figure 3C). As expected, the same effect is also observed for 3a. Despite 3d produces the lower amount of iROS in cells, it exhibits the same anticancer efficacy as the potent ROS generator 3a. These data indicate that, additionally to iROS, 3d might induce another intracellular toxicity factor. We found that this factor is mROS. In particular, the level of mROS (detected by using a MitoSOX™ probe) in A2780 cells treated either with the carrier or type A agent 3a for 2 h is very low as indicated by the weak red signal in fluorescence images E and I (Figure 3). Nuclei of the cells were counter-stained with a dye Hoechst 33342 (blue color) that was used as a reference signal for comparing changes in the intensity of the red fluorescence between different samples. In contrast to the control probes, the level of mROS in the 3d-treated cells is substantially higher as indicated by the intense red signal in image M. These qualitative microscopic data were fully confirmed by the accurate quantification of the intracellular mROS level using flow cytometry (Figure 3P). Since 3d itself is not a ROS amplifier (in contrast to 3a) as confirmed by the ROS-release experiment in cell free setting (Table 1), mROS should be generated indirectly. To get a hint on how this can happen, we investigated effects of 3d on mitochondria and lysosomes of cancer cells. Mitochondria were selected, since drug-induced disturbance of their function can lead to the inefficient electron transfer causing the electron leakage and formation of mROS. Lysosomes were selected, since 3d could be accumulated in these organelles due to the presence of a piperidine moiety (a lysosomal carrier) in its structure. By using mitochondrial membrane potential (MMP) sensitive probe rhodamine 123 (R123), we found that both 3d and 3a strongly reduce MMP in A2780 cells, whereas 2a is also active, but less efficient (Figure 4A). The effect of both type A agents 2a and 3a is saturated after 2 h incubation, whereas type B agent 3d is acting slower. In particular, the MMP of A2780 cells is decreased stronger after 4 h than after 2 h incubation with 3d (p < 0.01, Student’s t test). These data indicate that mROS generated by...

**Figure 3.** Uptake efficacy and involvement of ROS in the anticancer effect of agent 3d and Ref. [3a]. A: The efficacy of uptake of 3a and 3d by A2780 cells at 37 °C (black bars) and 4 °C (grey bars), determined by monitoring the increase of the intracellular iron concentration ([Iron]). B: Increase of iROS (responsive to a CM-DHFM-DA probe) in A2780 cells in the presence of 3a and 3d (each 25 μM, 2 h incubation), monitored by using flow cytometry: λex = 488 nm; λem = 570–640 nm. Student’s t test: * – p < 0.05; ** – p < 0.01; *** – p < 0.001; ns – non-significant, p > 0.05. Ref. references (color coded), against which Student’s test was applied.
3d can be caused by 3d-induced reduction of MMP in cancer cells.

Further, we found that agent 3d quenches the fluorescence of A2780 cells loaded with lysosome-specific probe acridine orange (AO). In contrast to the MMP, this effect is saturated already after 2 h incubation. These data might indicate that 3d is first accumulated in lysosomes before affecting the MMP. As already after 2 h incubation. These data might indicate that 3d is first accumulated in lysosomes before affecting the MMP. As already after 2 h incubation. These data might indicate that 3d is first accumulated in lysosomes before affecting the MMP. As already after 2 h incubation. These data might indicate that 3d is first accumulated in lysosomes before affecting the MMP. As already after 2 h incubation. These data might indicate that 3d is first accumulated in lysosomes before affecting the MMP. As already after 2 h incubation.

Intracellular species responsible for the anticancer effect of 3d

To clarify, which intracellular species 3d or 3d⁻ are responsible for the anticancer activity, we conducted a series of experiments in cell free settings and in cells.

Type B agent 3d is slightly more electron rich than unmodified ferrocene Fch: \( E_{1/2} (\text{vs Fch/Fch}⁺) = -35 \text{ mV} \). Accordingly, it catalyzes transformation of \( \text{H}_2\text{O}_2 \) to \( \text{HO}^\bullet \) 6.8-fold more efficiently than \( \text{Fch} \) (Table 1). In this reaction 3d donates an electron to \( \text{H}_2\text{O}_2 \) forming 3d⁻ at least in catalytic amounts. However, in HPLC of the mixture of 3d (100 \( \mu \text{M} \)) and \( \text{H}_2\text{O}_2 \) (10 \( \mu \text{M} \)) we observed only the peak corresponding to 3d. More polar 3d⁻, which was expected to elute at the shorter incubation, was not detected (Figure 5A, B). An analogous type B agent 2d is more electron rich than 3d: \( E_{1/2} (\text{vs Fch/Fch}⁺) = -70 \text{ mV} \). However, it is also not oxidized in the presence of \( \text{H}_2\text{O}_2 \) as it is evidenced from both HPLC (data not shown) and UV-visible spectroscopy: the absence of a typical for ferrocenium broad absorbance band at ~600–900 nm corresponding to the ligand-to-metal ("L→M") transition (Figure 5C, E).\(^{10}\) In another control experiment conducted under the same conditions, the "L→M" transition band at ~900 nm was observed in the spectrum of the mixture of type A agent 2a (560 \( \mu \text{M} \)) and \( \text{H}_2\text{O}_2 \) (10 \( \mu \text{M} \)) (Figure 5D). The intensity of this band steadily grows for over 50 min after addition of \( \text{H}_2\text{O}_2 \) to the solution of 2a (Figure 5E). The latter behavior was expected based on the previously reported mechanism of 2a activation by ROS via route I (Figure 1A). We further found that oxidation of type B agents is possible in the presence of a mixture of \( \text{H}_2\text{O}_2 \) and horseradish peroxidase (HRP), which is a stronger oxidant than \( \text{H}_2\text{O}_2 \) alone. This is indicated by the appearance of the "L→M" transition band in the UV-visible spectrum of the solution containing 2d, \( \text{H}_2\text{O}_2 \), and HRP (Figure 5C). 2d⁻ is formed almost instantaneously after the HRP addition 2d and \( \text{H}_2\text{O}_2 \) (Figure 5E).

For the study of possible oxidation of type B agents in live cells we prepared a fluorogenic analogue of 3d containing a 7-hydroxycoumarine dye – 7d. As controls we also prepared 6d, which is lacking a piperidine moiety (a fluorogenic version of 2d), and 6a (a representative fluorogenic type A agent). These compounds are practically not fluorescent due to the efficient photoinduced electron transfer (PET) from the ferrocenyl moiety to the excited state of the dye. Upon oxidation to the ferrocenium species, the fluorescence of 6a (via route I), 6d, and 7d (both via route II) is increased since PET becomes impossible. The experimental data illustrating this behavior for drug 7d are provided in Figure 5F. Similarly to its analogue 3d, drug 7d is practically not responsive to \( \text{H}_2\text{O}_2 \) whereas it is efficiently oxidized by a mixture of \( \text{H}_2\text{O}_2 \) and HRP that leads to the strong and quick increase of the fluorescence intensity.

By using fluorescence microscopy, we found that A2780 cells loaded with 7d exhibit weak, but significant fluorescence, whose intensity is not increased by the treatment of the cells with \( \text{H}_2\text{O}_2 \) (Figure 6A–C). In contrast, 6d-loaded A2780 cells were found to be non-fluorescent. However, by using flow cytometry, which is a more sensitive method, we observed that both 6d- and 7d-loaded A2780 and BL-2 cells fluoresce...
Figure 6. A–C Fluorescence images (λ_{em} = 335–383 nm; λ_{ex} = 420–470 nm) of A2780 cells incubated with fluorescent agents 6d (inset A), 7d (inset B) and 7d followed by H_2O_2 (inset C). [Drugs] = 25 μM; [H_2O_2] = 10 mM. The cells were first incubated with the agents for 2 h, washed and incubation for further 1 h with H_2O_2. D: Monitoring uptake/activation of 6a, 6d (both 25 μM) and 7d (1 μM) in A2780 (inset D) and BL-2 cells (inset E) by using flow cytometry: detection at λ_{em} = 405 nm; λ_{ex} = 405–495 nm. Student’s t test: * – p < 0.05; ** – p < 0.01; *** – p < 0.001; ns – non-significant, p ≥ 0.05. Ref: references (color coded), against which Student’s test was applied.

Figure 7. A: Tumor weigh in the studied groups at the end of the experiment. Statistic parameters were calculated by Mann-Whitney test. Dotted black line demonstrate the weight limit of 0.6 g, below which abundant granulomatous tissue was found in tumors. The area of large tumors is indicated with dotted red square. B–D: Histological analysis of selected tumors demonstrating blood vessels located deep inside tumor masses in the carrier (inset B); type A agent 3a (C) and type B agent 3d (D) groups. Blood vessels are indicated with green asterisks. Cell decay is indicated with yellow arrows. Tumor cells entering blood vessels space are shown with blue arrows. Anucleated areas are necrotic. H&E staining, 40×0.75 high-NA objective.

significantly stronger (p < 0.01 or less, Student’s t test) than the cells treated with DMSO only (Figure 6D, E). These data indicate that at least some 6d′ and 7d′ are formed within the cells from 6d and 7d correspondingly. This reaction cannot be triggered by H_2O_2 (compare B and C in Figure 6), but by some other oxidant.

Based on the reactivity of 3d and 7d in cell free settings (Figure 5), we assume that this oxidant can be an intracellular peroxidase or the oxidant with the “peroxidase-like” activity, for example Fe^{3+}/H_2O_2. A2780 and BL-2 cells loaded with type A agent 6a are stronger fluorescent than those loaded with type B agents 6d and 7d (Figure 6D, E). This indicates that 6a forms the higher amount of ferrocenium species in cells than 6d and 7d that is in agreement with properties of type A and B agents in cell free settings discussed above (Figure 5C, D, E).

To find out whether the ferrocenium species derived from 3d are important for the anticancer activity of this agent, we prepared its analogue brominated at the ferrocenyl fragment 3d_Br (Figure 1A). The redox potential of 3d_Br (E_{1/2} versus FcH/FcH^+) is 116 mV, which is by 151 mV higher than the redox potential of 3d. These data indicate that 3d_Br is more resistant towards oxidation than 3d. Therefore, we could expect that in cells 3d_Br will form the lower amount of the ferrocenium species than 3d. We observed that after 2 h incubation with A2780 cells 3d_Br produce similar amount of tROS, but less mROS than 3d (p < 0.05, Student’s t test). However, the effects of 3d_Br and 3d on the level of mROS at 4 h incubation were found to be the same. Further, we observed that cytotoxicity of 3d_Br and 3d towards A2780 and CLL cells are identical (Table 1, Figure 2C). Assuming that 3d_Br and 3d act via the same mechanism, these results allow concluding that the reduced form (3d) rather than the oxidized one (3d) are responsible for ROS-generating and anticancer properties of drug 3d.

Cancer cell specificity of agent 3d

We investigated cancer cell specificity of 3d in two experiments. First, by using fluorescence microscopy we compared the mROS level in representative cancer (human ovarian A2780 cells) and normal cells (human SBLF9 fibroblasts). These cells were loaded with 3d, incubated for 2 h, washed, loaded with MitoSOX^{TM} probe (mROS probe) and imaged. We observed the increased level of mROS in the cancer cells, but not in the normal cells (Figures 3D–O).

In the latter experiment we compared cancer and normal cells of different origins: human ovarian carcinoma versus human primary fibroblasts. Therefore, the effect observed is not necessarily caused by differences in cancer versus normal cellular phenotype. To address this issue, we compared anticancer effects of 3d towards cancer and normal cells originated from blood. They included chronic lymphocytic leukemia (CLL), B- and T-cells. We found that drug 3d kills both CLL and B-cells with similar efficacy, whereas it is substantially less toxic towards T-cells. These data indicate that 3d is not fully cancer cell specific. It can affect at least some normal cells thereby leading to the undesired toxicity.

Antitumor effects of 3d

We evaluated the anticancer activity of 3d in C57/BL6N mice carrying Nemeth-Kellner lymphoma (NK/Ly). The agent was formulated in cremophore/ethanol/saline (1/1/4, v/v/v) mixture. As a negative control we injected the mice with the carrier only. Furthermore, 3a formulated in the same carrier was used as a positive control. The agents and the carrier were applied at the dose of 8 mg/kg during the course of 10 injections, done every second day. The first injection was done one day after the tumor inoculation. On day 34, when the tumor weight in the carrier group was approaching 15% of initial animal weight, all animals were sacrificed, tumors were removed, weighted and fixed (Figure 7A). We observed that the average tumor weight in the 3a group was lower than that in the carrier group (p < 0.05, Mann-Whitney test). In contrast, 3d did not reduce the
By the optimization the chemical structure of type B aminoferrocene-based agent 1c we could substantially improve its anticancer efficacy towards human ovarian cancer A2780 cells from IC50 46.1 ± 8.5 μM for 1c to 7.0 ± 1.3 μM for the optimized agent 3d. We found that 3d is cytotoxic also towards other cells (Burkitt’s lymphoma BL-2 cell line and primary chronic lymphocytic leukemia CLL cells) in the low μMolar range. The anticancer efficacies of 3d and the best known type A agent 3a in vitro are comparable. 3d exhibits significant toxicity towards B-cells, whereas it does not affect T-cells and SBLF9 fibroblasts. Agents 3d and 3a acts differently from each other. In particular, the uptake of 3d occurs via the passive mechanism, whereas the uptake of 3a is energy dependent. 3d induces production of mitochondrial ROS (mROS), whereas 3a generates total ROS and no mROS. Agent 3d does not generate ROS directly as 3a does, but rather acts indirectly by reducing the mitochondrial membrane potential. By using the fluorogenic version of 3d, we observed that it is partially oxidized in cells with formation of ferrocenium species. However, an analogue 3d_Br, which has substantially higher redox potential than 3d (+151 mV), was found to have the same anticancer efficacy as 3d. These data indicated that the neutral form of the drug is responsible for its anticancer properties. Finally, we found that 3d exhibits moderate anticancer activity in vivo against murine Nemeth-Kellner lymphoma in C57/BL6N mice. Thus, this is the only AF-based agent, acting by increasing the level of intracellular mROS, which is applicable in vivo. All other known agents either exhibit low activity\(^\text{[11]}\) or are too toxic\(^{[10]}\).

**Experimental Section**

All experimental data, synthesis and assay protocols are provided in Supporting Information.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available in the supplementary material of this article.

**Keywords:** anticancer drugs · ferrocene · mitochondrion · reactive oxygen species

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