2,2'-Bipyridine-Modified Tamoxifen: A Versatile Vector for Molybdacarboranes

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Investigations on the antitumor activity of metalcarboranes are sparse in the literature and limited to a handful of ruthenium- and molybdacarboranes. In this study, the molybdacarborane fragment [3-(CO)\textsubscript{2}-closo-3,1,2-MoC\textsubscript{9}B\textsubscript{11}H\textsubscript{13}] was combined with a vector molecule, inspired by the well-known drug tamoxifen or 4,4'-dihydroxytamoxifen (TAM-diolOH). The molybdacarborane derivative [3,3-(1,1-bis(4-hydroxyphenyl)but-1-en-2-yl)-2,2'-bipyridine-k\textsuperscript{N,N}'-3(CO)\textsubscript{2}-closo-3,1,2-MoC\textsubscript{9}B\textsubscript{11}H\textsubscript{13}] (10), as well as the ligand itself 4(1,1-bis(4-hydroxyphenyl)but-1-en-2-yl)-2,2'-bipyridine (6) showed cytotoxic activities in the low micromolar range against breast adenocarcinoma (MDA-MB-231, MDA-MB-361 and MCF-7), human glioblastoma (LN-229) and human glioma (U-251) cell lines. In addition, compounds 6 and 10 were found to induce senescence and cytodestructive autophagy, lower ROS/RNS levels, but only the molybdacarborane 10 induced a strong increase of nitric oxide (NO) concentration in the MCF-7 cells.

Introduction

Medicinal chemistry is still dominated by purely organic molecules, but bioinorganic chemistry is becoming more and more important and several successful examples of metal-based drugs have already been reported (see special issue on Metals in Medicine in ACS Chemical Reviews).[1] However, the available biological data for molybdenum(II) complexes of the type [Mo(R)LX] (where R = (η\textsuperscript{3}~C\textsubscript{3}H\textsubscript{3})\textsuperscript{+}, (η\textsuperscript{3}~C\textsubscript{6}H\textsubscript{5})\textsuperscript{+}, η\textsuperscript{6}~C\textsubscript{6}H\textsubscript{6}) ; L = 1,10-phenanthroline, 2,2'-bipyridine, (CO)\textsubscript{9}, (MeCN)\textsubscript{9} ; X = Cl, BF\textsubscript{3} \textsuperscript{−} are very limited, and only a few studies were reported until now.[2] On the other hand, commercially available boron-based drugs are still rare, and mainly employed in boron neutron capture therapy (BNCT).[3] Unlike hydrocarbons, boranes and carboranes readily form clusters in a large variety of 3D geometric shapes. The icosaahedral closo-dicarbadodecaboranes or carboranes are highly interesting, due to their hydrophobicity, which is beneficial for transport across the blood-brain barrier (BBB), their inorganic nature preventing enzymatic degradation, their inherent low toxicity lowering side effects, their high boron content for BNCT, the variety of possible substitution patterns employing the three isomers (ortho-, meta-, para-carborane) and their 3D aromatic structures can be employed as bulky phenyl or cyclopentadienyl analogues.[4] Thus, a deboronation (i.e. formal loss of a B\textsuperscript{+} unit) of the closo-C\textsubscript{9}B\textsubscript{11}H\textsubscript{13} cluster and deprotonation, the so-called dicarbollide, [C\textsubscript{9}B\textsubscript{11}H\textsubscript{13}]\textsuperscript{−}, is obtained and can be employed in the preparation of a large variety of transition metal complexes resulting in full-, mixed- or half-sandwich structures.[5] Potential applications of the full-sandwich cobalt bis(dicarbollide) ([3,3-C\textsubscript{9}B\textsubscript{11}H\textsubscript{14}]\textsuperscript{2−}, COSAN) derivatives in medicine are well investigated,[6,7] but not as a pharmacophore. Also cytotoxic/cytostatic icosaahedral mixed-sandwich metalcarboranes, e.g. [3-(η\textsuperscript{5}-arene)-3,1,2-RuC\textsubscript{9}B\textsubscript{11}H\textsubscript{13}]\textsuperscript{−} have been reported.[8,9] Other half- and mixed-sandwich complexes with potential application in medicine have been summarized in the literature.[10] One advantage of half-sandwich metalcarboranes is the incorporation of biologically relevant ligands in the coordination sphere of the metals, as in the present study, where [3-(2,2'-bipyridine-k\textsuperscript{N,N}'-3(CO)\textsubscript{2}-closo-3,1,2-MoC\textsubscript{9}B\textsubscript{11}H\textsubscript{13}] (6) was equipped with a vector molecule which was inspired by the well-known tamoxifen. Further examples were reported by Causey et al.[11] Hawkins et al.,[12] Pruitt et al.[13] and Louie et al.[14] who used stable and hydrophobic rhenacarboranes including the [Re(L)\textsubscript{2}Cl\textsubscript{2}] fragment (L = 2,2'-bipyridine derivative or [NO\textsubscript{2}]) as imaging agents.

Nuclear receptor ligands using highly hydrophobic icosaahedral carborane clusters in structures like estradiol,[15] testosterone[15] or selective estrogen receptor modulators (SERMs)[16] were already reported. Also, a carborane-containing tamoxifen derivative, namely boroxifen, was synthesized, which showed, however, limited benefits.[17] Employing tamoxifen as lead structure is promising, because it exhibits estrogenic/antiestrogenic activity as well as cytotoxic properties,[18] and is also active in prevention of osteoporosis in postmenopausal women with early-stage breast cancer[19] and in the protection of cardiovascular functions.[20] Furthermore, its mode of action can be modified by small changes; e.g. an “A” ring substitution (Scheme 1) or modification with a nitro group in para position leads to aromatase inhibition (the enzyme producing estrogen).[21]
showed redox chemistry useful for the generation of reactive and extensively biologically tested by Jaouen derivative ferrocifen (Fc-diOH) (Scheme 1), which was reported

The synthesis of the 2,2'-bipyridine unit, which renders the ketone in compound 3 less reactive in this type of olefination reaction, unlike the electron-rich ferrocene derivative in the synthesis of ferrocifen.\textsuperscript{14} Equally unsuccessful was the use of 3 in a Wittig-Horner-type reaction. Therefore, ligand 6 was constructed stepwise starting from the commercially available 2-halo-isonicotinic acid (1, Scheme 2).\textsuperscript{15} A [Pd(PPh\textsubscript{3})\textsubscript{4}]\textsuperscript{-catalyzed Stille coupling reaction resulted in the asymmetric 2,2'-bipyridine derivative (3) in moderate to good yields (gram scale 56%, small scale 69%). A Horner-Wadsworth-Emmons modification for the Ramirez gem-dibromolefination gave compound 4 in good yield (78%).\textsuperscript{16} A [Pd(PPh\textsubscript{3})\textsubscript{4}]\textsuperscript{-catalyzed Suzuki coupling afforded compound 5 in good yield (76%). Demethylation of the aryl-methyl ether 5 with BBr\textsubscript{3} resulted in the final ligand 6.\textsuperscript{17} The conditions for the optimization of both Pd\textsuperscript{4}-catalyzed coupling reactions are given in the Supporting Information (Tables S1 and S2, SI). Compounds 3–6 were fully characterized by NMR, infrared spectroscopy, mass spectrometry, and elemental analysis; the solid-state molecular structure of 6 was also confirmed by single crystal X-ray diffraction methods (Figure S1, SI). After obtaining the new ligands, coordination studies were performed with [Ne\textsubscript{6}]\textsuperscript{3-} as the respective N,N-chelating ligand.

In the 4, 5, and 6 were referred by their respective acronyms N,N-chelating ligand, 3, and 3, 4, and 5, respectively. According to Schwarze.\textsuperscript{14} The compounds 8 and 10, respectively, bearing a simple 2,2'-bipyridine or 1,10-phenanthroline ligand,\textsuperscript{20} complexes 8–10 could be purified by flash column chromatography on silica gel in low to moderate yields (18–35%). These low yields are most likely related to a side reaction

Results and Discussion

Syntheses and Characterization

The synthesis of the 2,2'-bipyridine-substituted tamoxifen derivative (Scheme 2) via the facile McMurry coupling was not successful, probably due to the electron-poor 2,2'-bipyridine unit, which renders the ketone in compound 3 less reactive in this type of olefination reaction, unlike the electron-rich ferrocene derivative in the synthesis of ferrocifen.\textsuperscript{14} Equally unsuccessful was the use of 3 in a Wittig-Horner-type reaction. Therefore, ligand 6 was constructed stepwise starting from the commercially available 2-halo-isonicotinic acid (1, Scheme 2).\textsuperscript{15} A [Pd(PPh\textsubscript{3})\textsubscript{4}]\textsuperscript{-catalyzed Stille coupling reaction resulted in the asymmetric 2,2'-bipyridine derivative (3) in moderate to good yields (gram scale 56%, small scale 69%). A Horner-Wadsworth-Emmons modification for the Ramirez gem-dibromolefination gave compound 4 in good yield (78%).\textsuperscript{16} A [Pd(PPh\textsubscript{3})\textsubscript{4}]\textsuperscript{-catalyzed Suzuki coupling afforded compound 5 in good yield (76%). Demethylation of the aryl-methyl ether 5 with BBr\textsubscript{3} resulted in the final ligand 6.\textsuperscript{17} The conditions for the optimization of both Pd\textsuperscript{4}-catalyzed coupling reactions are given in the Supporting Information (Tables S1 and S2, SI). Compounds 3–6 were fully characterized by NMR, infrared spectroscopy, mass spectrometry, and elemental analysis; the solid-state molecular structure of 6 was also confirmed by single crystal X-ray diffraction methods (Figure S1, SI). After obtaining the new ligands, coordination studies were performed with [Ne\textsubscript{6}]\textsuperscript{3-} as the respective N,N-chelating ligand.

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which was not observed for the simple N,N-chelating ligands (e.g. 2,2'-bipyridine or 1,10-phenanthroline). During the purification process, more than one violet band could be observed for every complexation reaction. Exemplarily for 9, the second violet fraction was collected in sufficient purity and identified by \(^1\)H, \(^{13}\)C(\(^1\)H), \(^{11}\)B(\(^1\)H) and \(^{11}\)B NMR spectroscopy, HR-ESI mass spectrometry and diffraction methods as a chlorine B(8)-substituted derivative of 9 (see SI), where the respective coordination in the carborane ligand was activated for EINS-type (electrophile induced nucleophilic substitution) reactions (9b, Figure S2, SI). This one-pot two-step reaction (proton-mediated ligand exchange and EINS-type reaction at the dicar-bole) was attempted before without success for complexes of the 2,2'-bipyridine and 1,10-phenanthroline ligand, [3-(L-\(\kappa^N,s\)N')-3-(CO)]\(_2\)closo-3,1,2-MoC\(_2\)B\(_8\)H\(_{14}\)], with L = 2,2'-bipyrdidine or 1,10-phenanthroline, using different strong acids and solvents. Further studies for employing the chloride in carborane substitution chemistry are in progress.

The color of all the ligands ranges from colorless to pale yellow. On coordination to molybdenum(II), a color change to deep purple is observed for complexes 8–10 (for UV-Vis spectra of 6 and 10 see Figure S16, SI) indicating metal-to-ligand (MLCT) and ligand-to-metal charge transfer (LMCT) (with \(L = 3, 5\) or 6)\(^{[28]}\). In the infrared spectra, typical vibrations of all functional groups are observed, e.g. \(\nu(BH)\) (2595–2480 cm\(^{-1}\)) and \(\nu(CO)\) (1955–1874 cm\(^{-1}\)) vibrations, which are unique in biological systems and could, therefore, be used for label-free imaging (first attempts have been reported for [3-(2,2'-bipyridine-\(\kappa^N,N\)')-3-(CO)]\(_2\)closo-3,1,2-MoC\(_2\)B\(_8\)H\(_{14}\)]). In the \(^1\)H NMR spectra of the complexes 8, 9 and 10, there is a clear coordination shift observed compared to the chemical shifts of the ligands 3, 5 and 6 (Figure S3–S5, SI). The CH\(_{1\text{dioxo}}\) signals split into two broad singlets due to the asymmetric N,N-chelating ligand. Importantly, the coordination of 3, 5 and 6 at molybdenum(II) always generates two isomers, which cannot be distinguished via NMR spectroscopy. The carbonyl groups are observed as two slightly different signals at 255.3 and 254.5 ppm (CD\(_2\)Cl\(_2\)), 256.3 and 255.5 ppm (CDCl\(_3\)) or 260.3 and 260.0 ppm (CD, CN) in the \(^{13}\)C(\(^1\)H) NMR spectra of the respective complexes according to the C\(_2\) symmetry of the whole molecule.

Obtaining HR-ESI mass spectra of molybdacarboranes is challenging, since they cannot be ionized easily and are either detectable in negative or positive mode. Additionally, a strong clustering effect can be observed for complexes 8–10. For illustration, one typical high-resolution ESI mass spectrum in the negative mode is depicted in the supporting information (Figure S18, SI), showing an agglomeration pattern, where the unidentifiable agglomerate X (m/z = 1965.6786) loses five times a fragment [M-2H]\(^+$\). This effect can be minimized through dissolution of the sample immediately before injection into the ESI-MS instrument. This is a nice demonstration of the potential agglomeration properties of metallacarboranes in solution.

Bioanalytical Measurements

For in vitro cell culture tests, the stock solutions of sparingly water-soluble compounds are typically prepared in DMSO (ethanol or methanol are good alternatives) and stored below +4 °C. For that purpose, the chemical stability of 3, 5, 6 and 8–10 was tested in a solution of water-containing DMSO-d\(_6\) in air for at least 36 days (3, 5, 6, 8 and 9) and for 14 days for complex 10. \(^1\)H and \(^{11}\)B(\(^1\)H) NMR spectra revealed that the ligands 3, 5 and 6 can be stored in a DMSO stock solution for at least one month without decomposition, and the molybdacarboranes for 14 days up to one month with minor decomposition (where the decomposition products are the free ligands 3, 5 or 6, the nido-carborane ([nido-C\(_2\)B\(_8\)H\(_{12}\)]\(^-\)) and most likely a molybdenum species in higher oxidation states) (see Figure S6–S14). Recently, we introduced a formulation procedure for sparingly water-soluble metallacarboranes, using fatty acids and magnesium-free bovine serum albumin (BSA\(_{\text{noMg}}\)) in a 1:10 molar ratio, which led to a significant improvement of the reproductibility, but also the cytotoxicity against MCF-7 breast cancer cells. Here, the biological activity of molybdacarboranes (8–10), as well as ligands 3, 5 and 6, and literature-known reference compounds (TAM-diOH and Fc-diOH) was evaluated employing this formulation procedure to ensure comparability.

To understand the self-assembling behavior of metallacarboranes/BSA\(_{\text{noMg}}\) co-assemblies, exemplarily ligand 6 and complex 10 were investigated in phosphate-buffered saline (PBS)/DMSO mixtures at physiological pH (pH 7.4) by UV-Vis, fluorescence and Rayleigh light scattering (RLS) spectroscopy, as well as Nanoparticle Tracking Analysis (NTA)\(^{[9,29,35]}\). For the fluorescence spectra, three excitation wavelengths were chosen (\(\lambda_{\text{exc}} = 280, 295\) and 320 nm) and the investigations were performed with two site markers, namely warfarin and ibuprofen, binding selectively to Sudlow’s site I or Sudlow’s site II, respectively. The same systems were investigated by UV-Vis spectroscopy in parallel. Our measurements revealed that 6 binds to both, Sudlow’s site I and II (Figure S15, SI).

Remarkably, the fluorescence intensity at \(\lambda_{\text{exc}} = 280\) nm is quenched to the same extent when 6 only is mixed with
BSA$_{\text{noMg}}$ or together with ibuprofen, indicating that the binding strength is in the same order of magnitude as for the site marker. The situation is slightly different for 10, which binds to Sudlow’s site II, but the fluorescence is quenched more in the ternary system together with BSA$_{\text{noMg}}$ and ibuprofen. The fluorescence intensity is quenched more when 10 is added first, and ibuprofen afterwards, which might imply a weak cooperative effect upon binding of both components (Figure S15, SI). Evidently, there is a strong cooperative effect of both 6 and 10 with warfarin (at the excitation wavelengths $\lambda_{\text{exc}} = 295$ and 320 nm), which is independent from the order of addition. Also, tamoxifen acts differently, having a negative allosteric effect on warfarin—HSA binding.

These findings are in contrast with the ones for the molybdacarborane $[3-(2,2'$-bipyridine-$\kappa^2N,N')$-3-$(\text{CO})_2$-closo-3,1,2-MoC$_6B_9H_12]$ (i), which seems to bind stronger to Sudlow’s site I than to site II, without any interaction being detected between warfarin and i.

The absorption spectra of 6 in PBS/DMSO mixture is essentially the same as for fluorescence spectra. Notably, the UV-Vis spectrum of the same overall trend concerning binding events as the spectrum suggests that the solubility of the ternary systems (BSA-site marker-drug, Figure S16, SI) show (CO) as detected between warfarin and i.

In vitro Cell Colorimetric Assays

After we found evidence of cytotoxicity of $[3-(2,2'$-bipyridine-$\kappa^2N,N')$-3-$(\text{CO})_2$-closo-3,1,2-MoC$_6B_9H_12]$ (i) against MCF-7 cells, we wanted to expand our studies to other molybdacarboranes with N,N-chelating ligands and a larger panel of cell lines, i.e. three breast cancer adenocarcinomas (MDA-MB-231, MDA-MB-361 and MCF-7), human glioblastoma (LN-229) and human glioma (U-251) cell lines, plus mouse macrophages (MF), as an example for non-malignant immune cells (Table 1 and Figures S19 and S20, SI). Cells were exposed to 3, 6 and 8–10 for 72 h, after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet (CV) cell viability assays were performed.

As we found an improvement of the cytotoxicity for i upon formulation with BSA$_{\text{noMg}}$ in a 1:10 ratio (compound:BSA$_{\text{noMg}}$) in previous studies, we performed all studies presented here with the mentioned formulation protocol, also for the non-metalcarborane compounds 3 and 6, as well as the two reference compounds 1,1-bis(4-hydroxyphenyl)-2-phenylbut-1-ene (TAM-diOH) and ferrocifen (Fc-diOH). Under the applied conditions of polydispersity (Figure 1, right) being stable over 20 h, supporting the findings from previous studies.

Interestingly, when the aggregation behavior of only the ligand 6 is investigated, no such effect is observed. Indeed, the very broad size distribution of particles of 6 in PBS/DMSO is transformed into a size distribution that resembles BSA in PBS/DMSO (Figure 1, left) implying that 6 is bound to BSA (see also spectroscopic data: Figure S15–S16, SI), but does not induce agglomeration as metallacarboranes do. Thus, these findings are a strong evidence that metallacarboranes form nano-sized aggregates with BSA$_{\text{noMg}}$ which cannot be observed for organic hormone-like structures, even though binding to BSA$_{\text{noMg}}$ was proven for 6 via UV-Vis and fluorescence spectroscopy.
the biological evaluation, 5 was not suitable for testing due to precipitation from the solution, and 9b could not be obtained in sufficient purity. The results revealed that ligand 3 and the corresponding metallacarborane 8 have moderate to low cytotoxic activity on the studied cell lines. In all cases, except for the MCF-7 cells, the IC50 values for molybdacarbone complexes are slightly higher than for the respective ligands alone. Complex 9, bearing a vector which is closer to the lead structure of TAM-diOH, performs expectedly better, especially against the MCF-7 cell line (Table 1). Remarkably, also the human glioblastoma cells (LN-229) and human glioma cells (U-251) were sensitive towards treatment with 9 in the low/ moderate micromolar range. The couple 6 and 10 showed activity against all tested cell lines in the low micromolar range (Table 1). Worth highlighting is that the substitution of the phenyl ring “A” in TAM-diOH by a chelating 2,2’-bipyridine unit does not only improve the anticancer activity for all tested breast cancer cell lines, but also provides cytotoxic activity against the very aggressive primary malignant glioblastoma cell line by flow cytometry. Fc-diOH was also tested, as reference, to evaluate a possible mode of action of the new compounds, 6 and 10 were selected for further analysis on the MCF-7 cell line by flow cytometry. Fc-diOH was also tested, as reference, for comparison. All following investigations were performed applying the BSAmedium formulation protocol.

Both 6 and 10 showed the same moderate inhibitory properties on cell proliferation (carboxyfluorescein succinimidyl ester (CFSE) staining, Figure 2A), whereas Fc-diOH featured stronger inhibition of the proliferation, in line with the literature for all ferrocen-type anticancer drugs.85 Senescence-associated (SA) β-galactosidase staining revealed that 6 induces senescence to a minor extent compared to the transition metal-containing 10 and ferrocen (Figure 2B).89

Cellular senescence is an important mechanism to prevent tumor cell proliferation and might result in cellular death.40 However, the detailed role of senescence in general was found to be way more complicated, because this process not only arrests the cell cycle, but it also stimulates the cells to produce numerous growth factors and other proteins (also called senescence-associated secretory phenotype, SASP), which can, in turn, provoke again tumor promotion but also tissue repair.41 Annexin-V/PI double staining indicates that apoptosis is not the main mode of action for either 6 or 10 (Figure 2C), which is the case for the reference compounds and for ruthenacarboranes.82,83

These results could be supported by 4,6-diamidino-2-phenylindole (DAPI) staining, highlighting shrunken nuclei with condensed chromatin as sign of apoptosis (Figure 2D, red

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**Table 1.** IC50 values for 3, 6, 8–10 from MTT and CV cell viability assays. Standard deviations for each IC50 value are given.

| Compound | Assay | MDA-MB-231 | MDA-MB-361 | MCF-7 | U-251 | LN-229 | MF |
|----------|-------|-------------|-------------|--------|--------|--------|----|
| 3        | MTT   | 42.0 ± 3.4  | 78.1 ± 3.8  | 43.0 ± 2.9 | 38.0 ± 3.0 | 47.2 ± 0.9 |    |
|          | CV    | 38.1 ± 3.5  | 70.5 ± 5.2  | 41.3 ± 2.2 | 44.1 ± 1.6 | 54.9 ± 4.1 | > 100 |
| 6        | MTT   | 2.2 ± 0.2   | 2.4 ± 0.2   | 1.8 ± 0.4  | 15.2 ± 0.6 | 15.8 ± 2.0 | > 100 |
|          | CV    | 2.5 ± 0.2   | 2.8 ± 0.3   | 2.1 ± 0.6  | 21.0 ± 3.5 | 17.7 ± 1.0 | > 100 |
| 8        | MTT   | > 100       | 71.0 ± 4.4  | 34.4 ± 1.7 | 97.4 ± 3.7 | > 100    | > 100 |
|          | CV    | > 100       | 75.7 ± 5.0  | 27.9 ± 3.5 | 55.8 ± 5.1 | > 100    | > 100 |
| 9        | MTT   | 38.2 ± 1.4  | 18.3 ± 1.4  | 6.0 ± 0.6  | 17.2 ± 0.6 | 21.9 ± 1.6 | > 100 |
|          | CV    | 30.8 ± 2.8  | 18.1 ± 2.4  | 6.6 ± 0.9  | 17.4 ± 1.0 | 25.8 ± 1.7 | > 100 |
| 10       | MTT   | 17.8 ± 2.0  | 4.0 ± 0.4   | 9.3 ± 0.5  | 30.1 ± 2.8 | 38.0 ± 0.9 | > 100 |
|          | CV    | 18.5 ± 1.1  | 4.5 ± 0.6   | 5.0 ± 0.7  | 32.7 ± 3.8 | 41.0 ± 3.8 | > 100 |
| TAM-diOH | MTT   | 15.4 ± 0.6  | 24.5 ± 3.6  | 24.3 ± 1.6 | > 100    | > 100    | > 100 |
|          | CV    | 16.8 ± 1.3  | 33.9 ± 1.3  | 25.3 ± 2.4 | > 100    | > 100    | > 100 |
| Fc-diOH  | MTT   | 1.0 ± 0.1   | 1.5 ± 0.3   | 1.0 ± 0.1  | 1.4 ± 0.2 | 3.1 ± 0.2 | < 100 |
|          | CV    | 0.9 ± 0.1   | 2.3 ± 0.2   | 1.0 ± 0.1  | 1.7 ± 0.1 | 2.6 ± 0.3 | 20.6 ± 0.6 |
arrows), in the case of 
10 to a lesser extent than for 
Fc-diOH. However, for 
6 a prevalent observation is the presence of irregular-shaped shrunken nuclei (Figure 2D, yellow arrows) indicating sporadic cells with apoptotic morphology. In concordance with the data above, the activation of caspases was only detected upon treatment of MCF-7 cells with Fc-diOH (Figure 2E).

Additionally, autophagy processes were triggered by all three tested compounds (Figure 3A); however, different modes of action could be detected after treatment with the autophagy inhibitor 3-methyladenine (3-MA). Since Fc-diOH further lowers the cell viability when applied in combination with 3-MA, the autophagy process can be considered as cytoprotective (pro-survival) (Figure 3A, bottom right panel), while the addition of 6 as well as 
10 together with 3-MA resulted in restored viability compared to 6 (or 10) alone, and it can therefore be regarded as cytodestructive (cell death mediating, Figure 3A, upper right and bottom left panel). This non-apoptotic and autophagy-mediated cell death mechanism could be the pathway of how 6 and 
10 act on the tested cancer cells. In the literature, also autophagy is discussed as a double-edged sword, depending on healthy or cancer cell type, microenvironment, and development status.[43]
While in the luminal breast cancer cell line MCF-7 autophagy seems to be a reasonable cell death process, it was recently shown for other common chemotherapeutics that autophagy in e.g. MDA-MB-231 cells is dependent on other signaling pathways.\[44\] Autophagy, as one of the main cellular processes, can have a large spectrum of physiological functions affecting cell fate in various ways. Its specific role, however, is determined by the cell specificity, the intensity of triggered processes, as well as the type of the chemotherapeutic that induced it.\[45\]

To investigate the capability of the drugs to generate reactive oxygen/nitrogen species (ROS/RNS), dihydrorhodamine 123 (DHR 123) staining was performed. It could be shown for Fc-diOH that the ROS/RNS levels in the treated cells were increased, as it is reported in the literature via the inhibition of cytoprotective radical scavenger thioredoxin,\[46\] as well as the self-generation of ROS/RNS species, which all together lead to apoptosis also in hormone-independent cell lines.\[22\] Similarly, other ferrocene-containing bioactive molecules induce apoptosis through the generation of ROS.\[23,47\] In contrast, both 6 and 10 “scavenge” or lower the cellular ROS/RNS levels (DHR 123, dihydrorhodamine 123 staining; Figure 3C), which corresponds well with the absence of caspase-dependent apoptosis induction. Worth mentioning is that the molybdacarborane 10 is way more efficient in scavenging/reducing radical concentration compared to its ligand (6) only. How exactly 6 and 10 lower the cellular ROS/RNS levels remains unclear and will be addressed in future studies.

Remarkably, compound 10 strongly upregulates the NO production in MCF-7 cells (Figure 3B). Either it scavenges ROS/RNS and thus generates NO, or it interacts with one or more of the nitric oxide synthases (NOS), as known for other mixed-sandwich ferracarborane or COSAN-type structures.\[7\] This effect

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**Figure 3.** Flow cytometry analysis of MCF-7 cells exposed (72 h) to an IC\(_{50}\) dose of 6, 10 or Fc-diOH, respectively, applying the BSA\(_{noMg}\) formulation procedure. (A) AO staining (upper left panel), fluorescence intensity from the FL3 channel (dark red emission) for detection of acidic vacuoles; cell viability of MCF-7 cells was determined via CV assays upon treatment with 3-MA, 6, 10 or Fc-diOH and 3-MA with 6, 10 or Fc-diOH. Experiments were run in triplicate. The respective control (cells treated with BSA\(_{noMg}\)) is also shown. p < 0.05 in comparison to * non-treated cells or # treated with the respective compounds alone (6, 10 or Fc-diOH). (B) DAF-FM (4-amino-5-methylamino-2,7-difluorofluorescein) staining allows to detect the nitric oxide (NO) level, fluorescence intensity from the FL1 channel; (C) DHR 123 staining for determination of the ROS/RNS species, fluorescence intensity from the FL1 channel (green emission). For flow cytometry, experiments were run in triplicate. The representations are based exemplarily on one experiment. For each staining protocol, the respective control (cells treated with BSA\(_{noMg}\)) is also shown.
is in high contrast to the other two tested compounds (6 and Fc-diOH) in flow cytometry analysis. Also, nitric oxide has an ambivalent role in healthy and cancer cell correlation. In low concentrations, i.e. 10 – 300 nM, proliferation of MCF-7 cells could be induced via HIF-1α (hypoxia-induced factor 1α), whereas in higher concentrations, i.e. > 300 nM, the p53 tumor protein is further phosphorylated inducing an apoptotic cell death mechanism.

Conclusions

We could synthesize a 2,2’-bipyridine vector (6) in five synthetic steps that can be employed as ligand for a large variety of organometallic or inorganic transition metal complex fragments giving distinct functions to the whole complex. The activity of the prototypic molybdacarborane [3-(2,2’-bipyridine-x’N,N’)-3-(CO)2-closo-3,1,2-Mo2C6B6H10] (II), already active against MCF-7 breast cancer cell line (IC50 = ca. 40 μM), could be improved by incorporating the 2,2’-bpy-TAM-diOH (6) ligand. Furthermore, the ligands 3 and 6 were tested in cell viability studies (CV and MITT assays) in comparison to the respective molybdacarborane complexes (8–10) against breast adenocarcinoma (MDA-MB-231, MDA-MB-361 and MCF-7), human glioblastoma (LN-229) and human glioma (U-251) cell lines and showed IC50 values in low to moderate micromolar range. Importantly, viability of mouse macrophages (Mφ) was not significantly affected by the new compounds up to a concentration of 100 μM, in contrast to Fc-diOH. Modification of the “A” ring in 4,4’-dihydroxytamoxifen (TAM-diOH) or ferrocifen (Fc-diOH) with 2,2’-bipyridine or [3-(2,2’-bipyridine-x’N,N’)-3-(CO)2-closo-3,1,2-Mo2C6B6H10] modulates the anticancer activity profile significantly. Compounds 6 and 10 were found to have distinctly diverse activities (compared to Fc-diOH), e.g. induction of senescence, cytotoxic destructive autophagy, lowering of ROS/RNS levels, and, uniquely for 10, strong increase of nitric oxide (NO) concentration. In perspective, it might be a good chance that the tested compounds are active on different routes rather than causing apoptosis, because most anticancer drugs kill through apoptotic cell death, thus having only little effect on cancer cell lines with defects in the classical apoptotic signaling pathway. Further details on the mode of action of 6 and 10 are under way.

This study shows that half-sandwich molybdacarboranes are highly interesting bioactive pharmacophores with distinct activity profile, which can modulate the mode of action of established organic and organometallic anticancer agents. Additionally, it could be shown via Nanoparticle Tracking Analysis (NTA) that the molybdacarborane unit is essential for triggering the formation of nanoparticles upon preparation with bovine serum albumin (BSA~nano~), which might have a positive impact on in vivo studies. Thus, both, 6 and 10, are promising drug candidates for application in breast cancer, glioma and glioblastoma therapy.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: breast cancer · molybdacarborane · tamoxifen · nitric oxide (NO) · nanoparticles

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