A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

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Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201703492
Angew. Chem. 10.1002/ange.201703492

Link to VoR: http://dx.doi.org/10.1002/anie.201703492
http://dx.doi.org/10.1002/ange.201703492

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Modular Assembly of Reversible Multivalent Targeting Drug Conjugates

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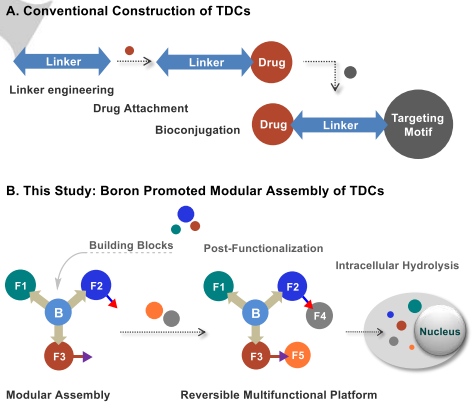
Abstract: Here is described a new modular platform to construct cancer cell targeting drug conjugates. Tripodal boronate complexes, featuring reversible covalent bonds, were design to accommodate, a cytotoxic drug (bortezomib), polyethylene glycol (Peg) chains and folate targeting units. The B-complex core was assembled in one step, and proved stable in different biocompatible conditions, namely human plasma (half-life up to 60 h) and reversible in the presence of glutathione (GSH). The stimulus responsive intracellular cargo delivery was confirmed by confocal fluorescence microscopy and a mechanism for GSH induced B-complex hydrolysis was proposed based on mass spectrometry and DFT calculations. This platform enabled the modular construction of multivalent conjugates exhibiting high selectivity for folate positive MDA-MB-231 cancer cells and IC_{50}'s in the nanomolar range.

Recent developments of human biology facilitate a more clear understanding of the intricate pathogenesis of complex diseases such as cancer.[1] The evolution of normal cells to a neoplastic state is a multifaceted biological process in which normal cells acquire capabilities of sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis.[1a] Therefore the most recent strategies to tackle cancer, aim at interrupting one or more of these stages using multifunctional constructs, in which, the biological activity of the individual components is preserved and tuned depending on the construct structure. Despite conceptually simple, the assembly of multifunctional constructs is often hampered by the overwhelming complexity of the synthetic process.[2]

Targeting drug conjugates (TDCs), like antibody (ADCs) and small-molecules drug conjugates (SMDC), are multifunctional constructs that combine the lethality of potent cytotoxic drugs with the targeting ability of specific biomolecules that elicit a high affinity for antigens overexpressed in cancer cells.[3] In these conjugates, the linker technology used to connect both functions contributes decisively for the therapeutic usefulness of these constructs, by enabling the biomolecule functionalization without altering its pharmacokinetic properties, by maintaining the conjugate integrity in circulation and triggering the release of the active drug only upon reaching the target.[4] Given these requirements, the linker engineering often contemplates the incorporation of reactive handles to selectively attach the drug and the biomolecule, groups that may control the conjugate physicochemical properties (e.g. Peg) and cleavable units that promote the stimulus responsive (e.g. enzymes, pH, GSH) release of the cargo at the target.[4] Hence, linkers often exhibit structures with a high functional density, and their construction involves a series of complex and costly synthetic steps that are typically unsuited for a straightforward structural diversification (Scheme 1A).[4b,c] Therefore, the engineering of composite multifunctional molecules that enable the tuning of the construct properties by simple variation of the components individual properties, are highly desirable for the expedient discovery of new TDCs with therapeutic usefulness.[4b,c]

Boronic acids (BAs) readily establish reversible covalent bonds with Schiff base ligands to yield boronate complexes (B-complexes), featuring a modular and reversible tripodal framework.[5] Based on this, we conceive that if B-complexes would display suitable properties of stability and controlled reversibility in biological settings, they could be used as a platform to design multifunctional constructs to selectively target and deliver cargo to cancer cells (Scheme 1B).



Scheme 1. (A) Linear construction of TDCs; (B) Modular and reversible assembly of multifunctional TDCs promoted by boron.

To test this idea, we prepared several B-complexes based on a one-pot three-component reaction in which 4-methoxysalicylaldehyde and phenyl BA were reacted with L-phenylalanine, anthranilic acid or 2-aminophenol in equimolar amounts. This simple protocol, that requires no chromatographic isolation step, afforded the corresponding complexes **1**, **3** and **4** in yields up to 90 % (Scheme 2, see also sections 1.2-1.4 SI). Once prepared, the stability of these cores was evaluated at physiological pH 7.4 and compounds **1**, **3** and **4** displayed half-lifes lower than two hours in this media (Scheme 2, see also sections 2.2, 2.4 and 2.5 SI). Inspection of the boronated cores suggested that the poor stability of these molecules could be due to a rapid hydrolysis promoted by the addition of water to the electrophilic imine C-atom.[6] Consequently, to overcome this excess of reactivity, analogous complexes were synthesized starting from 2-hydroxy-4-methoxyacetophenone. This proved to

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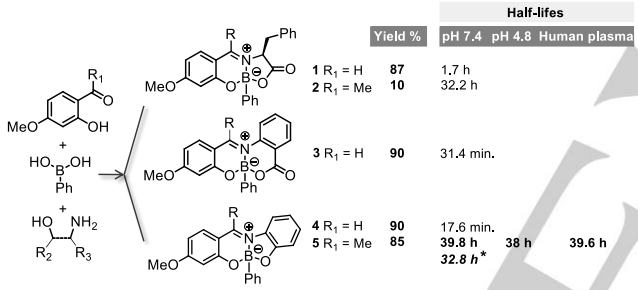
be a slightly more challenging transformation and, despite our many attempts, the preparation of the complex from anthranilic acid was not achieved. Despite this, the introduction of a methyl substituent on the imine C-atom of **2** and **5** (Scheme 2, see also sections 1.2 and 1.4 SI) drastically improved their stability at pH 7.4, in particular B-complex **5**, prepared from 2-aminophenol, displayed a half-life of 39.8 h and this performance was maintained at a lysosomal pH 4.8 ($t_{1/2}$ = 38 h) and in human plasma ($t_{1/2}$ = 39.6 h) (Scheme 2, see also section 2.6-2.8 SI).

After establishing the stability of these cores in different biological conditions, we studied their stimulus responsive hydrolysis. Cancer cells typically exhibit an increased concentration of GSH (micromolar range) in the cytoplasm, and for that reason, GSH has been extensively targeted to promote conjugates dissociation upon internalization.[7] Based on this, the stability of **5** at pH 7.4 was studied in the presence of 10 equiv. of GSH. As expected, the presence of GSH compromised the structural integrity of the B-complex and the half-life of **5** was reduced by 7 h in these conditions (Scheme 2, see also section 2.10 SI). Taking in consideration the stability and controlled reversibility exhibited by B-complex **5**, hydroxyacetophenone and aminophenol were selected as the components to test the assemblage of a multifunctional TDC featuring a cytotoxic drug, a small Peg chain and a targeting unit.

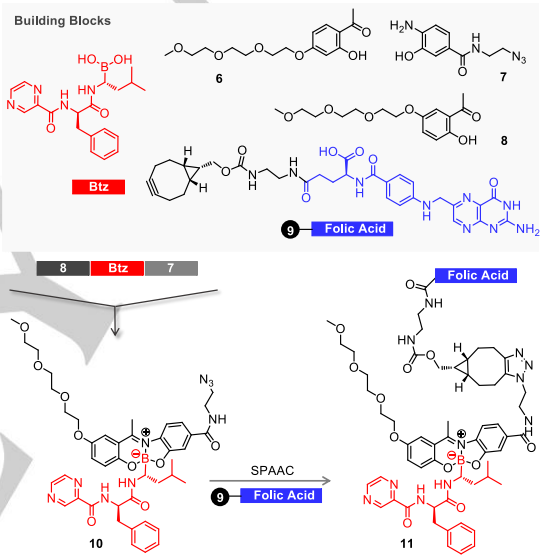


Scheme 2. One pot synthesis of B-complexes **1**-**5** and yields obtained. Evaluation of their stability at pHs 7.4, 4.8 and in human plasma. * B-complex **5** was also evaluated at pH 7.4 in the presence of 10 equiv. of GSH.

Bortezomib (**Btz**) is a potent proteasome inhibitor approved by FDA for the treatment of myeloma and lymphoma cancers. However, this drug is far from reaching its true therapeutic potential, because the BA function readily forms reversible covalent complexes with vicinal Lewis base donors of proteins, peptides and sugars, which contributes to the emergency of unfavourable pharmacokinetic properties and off-target toxicities.[8] Therefore, **Btz** was selected as the cytotoxic component, not only because it exhibits a BA function that is essential to generate the B-complex, but also because **Btz** would benefit from an improved selectivity endowed by the construct vectorization to cancer cells.

Regarding the targeting unit, folic acid was chosen to direct the SMDCs to cancer cells. Folic acid is an essential vitamin for cell functioning and is commonly used as a recognition moiety, because many cancer cell lines over-express receptors for this small vitamin due to their fast cell division and growth.[9]

Next, we addressed the preparation of the remaining components of the boronated core. Following reported methodologies, the hydroxyacetophenone and the aminophenol components were modified to incorporate a small Peg chain (**6**) and an azide function (**7**) for post-functionalization with a folate-cyclooctyne unit (**9**) (Scheme 3, see also sections 1.5-1.7 SI).[10] With all components at hand, the assembly reaction was attempted using **Btz**, **6** and **7**, though the desired product was never formed. We reasoned this lack of reactivity to be due to a combination of stereo constraints imposed by the **Btz** structure and the Peg chain in **6** (see section 6.1 SI). Consequently, the 4-substituted hydroxyacetophenone **8** was synthesized and reacted with **7** and **Btz**. Pleasingly, these components readily afforded **10**, which was isolated in 25 % yield. Next, **9** was used to install the folate unit directly onto the B-complex **10** *via* a strain promoted alkyne-azide cycloaddition – SPAAC. The reaction proceeded smoothly in DMSO and the formation of **11** was confirmed by ESI-MS (Scheme 3, see sections 1.8-1.9 SI).

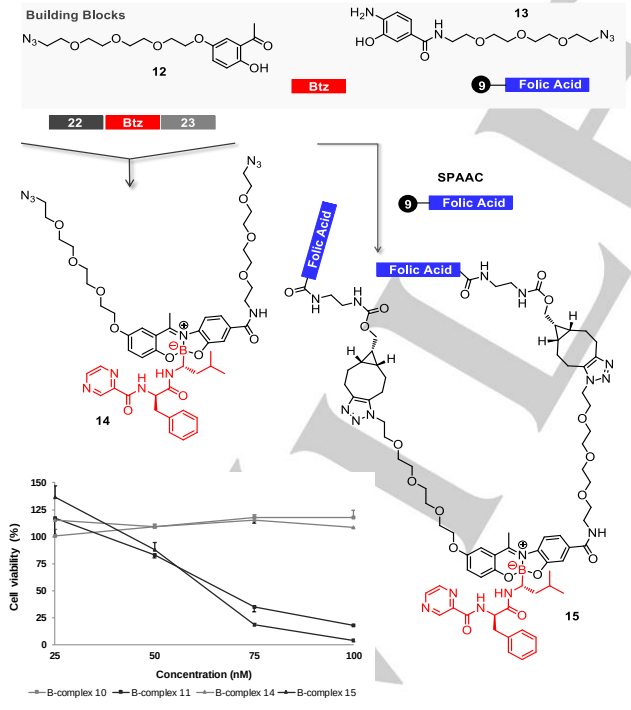


Scheme 3. Assemblage of B-complex **10** (CH_3CN, 75 ºC, 18h, 25 %) and subsequent folate functionalization via SPAAC (DMSO, 25 ºC, 17 h, 85 %) to yield compound **11**.

Once prepared, the selectivity and cytotoxicity of B-complex **11** was evaluated against MDA-MB-231 human breast cancer cells that overexpress folate receptors (FR), using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl)-2H-tetrazolium (MTS) cell proliferation assay. The study started with the evaluation of **Btz,** which was shown to be quite cytotoxic (IC_{50} of 14.2 nM) against this cancer cell line. Next, the cytotoxicity of B-complexes **10** and **11** was determined in the same conditions. In this assay, **10** was completely inactive up to a concentration of 100 nM while B-complex **11**, featuring a folate targeting unit, exhibit an improved potency (IC_{50} of 67.5 nM) against this cancer cell line (Scheme 4, see also section 3.2.1 SI). Considering that the free folic acid **9** was also non cytotoxic against MDA-MB-231 cancer cells (see section 3.2.1 SI), the observed activity of **11**, clearly suggests that folic acid is mediating the B-complex internalization into the cancer cell where **Btz** is released. Encouraged by these results, the platform modularity was explored to tune the construct structure and activity.

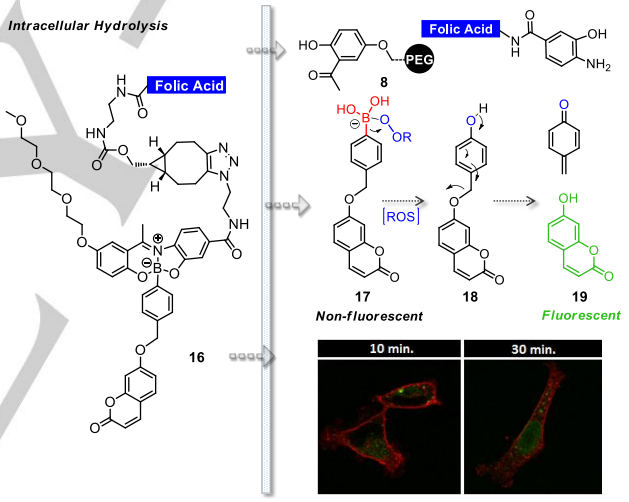
Antibody (Ab) drug conjugates are a powerful class of TDCs that use Abs (e.g. Immunoglobulin G - IgG) as the tumor cell recognition moiety.[4a] IgGs typically feature bivalent Fab regions that are responsible for the high specificity and affinity of the Ab against a determine epitope. Inspired by this design, we envisioned that the boronated core could also be modified to install a bivalent folate recognition moiety using hydroxyacetophenone **12** and aminophenol **13** featuring small Peg chains and azide functions.[11] As shown in Scheme 4, **12** and **13** were readily synthesized and used in the successful assembly of B-complex **14** that was isolated in 8% yield (see also sections 1.10-1.12 SI). The post-functionalization of **14** with the folate-cyclooctyne **9** afforded B-complex **15** (see section 1.13 SI), which was then tested against MDA-MB-231 cancer cells. As shown in Scheme 4, the incorporation of a bivalent folate recognition moiety maintained the conjugate **15** ability to deliver **Btz** and a IC_{50} of 62 nM (Scheme 4, see also section 3.2.1 SI).

To determine the selectivity induced by the presence of the folic acid targeting unit, both B-complexes **11** and **15** were tested against a 4T1 mouse breast carcinoma cell line with a very low expression of folate receptors. Differently from **Btz** that proved to be highly cytotoxic against this cell line (IC_{50} of 22.8 nM), both constructs did not perturb the cells viability in up to a concentration of 100 nM, being only cytotoxic at higher concentrations (1-100 µM) (see section 3.2.2 SI). These results indicate that B-complexes **11** and **15** are somewhat less potent than **Btz** though, comparing with the free drug, both exhibited an improved selectivity towards overexpressed FR cell lines.



Scheme 4. Construction of B-complex **14** (CH_3CN, 75 ºC, 18 h, 8 %). Folate functionalization *via* SPAAC (DMSO, 25 ºC, 17 h, 99 %) to yield difolic SMDC **15**. Cell viability determined by MTS assay, after 48h incubation of MDA-MB-231 (ATCC® HTB-26TM) with **Btz** (5-100 nM), and B-complexes **10**-**11**, **14**-**15** (25-100 nM).

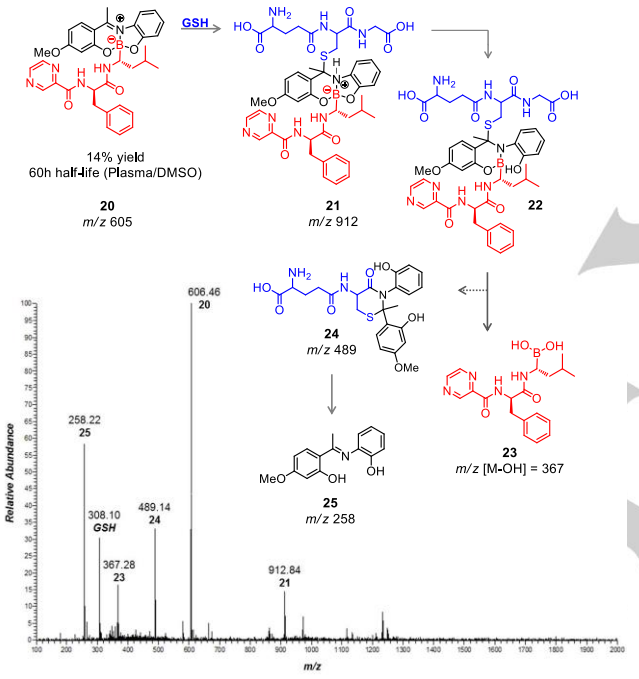
After establishing the potential of this molecular arrangement to engage in an effective targeted delivery of **Btz**, we studied the drug delivery mechanism. Hence, B-complex **16** featuring a non-fluorescent coumarin substituted phenyl BA was prepared (see section 1.14 SI) and used to label MDA-MB-231 and 4T1 cancer cells (Scheme 5). It is well established that BAs readily undergo oxidative boronate cleavage in the presence of intracellular reactive oxygen species, namely at cancer cells' cytoplasm.[12] Hence, B-complex **16** was designed to signal the hydrolysis of the BA component *via* the oxidation of the BA to phenol that self-emotively generates the quinone methide and releases the fluorescent coumarin probe as shown in Scheme 5. To test this hypothesis, **16** (1 µM) was added to MDA-MB-231 and 4T1 cells and incubated for 10, 30 and 180 minutes. Images obtained by confocal microscopy clearly show in MDA-MB-231 cells, the fluorescent coumarin probe distributed throughout the cell cytoplasm, and confirmed the successful B-complex **16** entry into the cells, as well as the hydrolysis of the BA component already at the first time point recorded (Scheme 5, see also section 4 SI). Gratifyingly, a similar internalization pattern was not observed when using the 4T1 cells (section 4 SI).



Scheme 5. Synthesis of complex **16** and confocal fluorescence microscopy analysis of its incubation in MDA-MB-231 human breast cancer cells. Plasma membrane was labelled with WGA-Alexa Fluor® 594.

Upon confirming the BA release inside the cancer cell, GSH was studied as the promoter of the B-complex disassembling. With this objective, **20** was prepared (see section 1.15 SI) and evaluated in plasma (see section 2.9 SI). This compound proved to be more stable than the analogue **5** constructed with phenyl BA, and exhibit a half-life of 60 h in these conditions. Next, B-complex **20** was incubated with GSH in ammonium acetate buffer pH 7.4 at 37 ºC and the reaction was monitored by ESI-MS over 72 h (see section 5 SI). As shown in Scheme 6 (see section 5 SI), analysis of the reaction mixture immediately indicated the presence of an adduct combining the masses of **20** and **GSH** (*m/z* 912, Scheme 6). Signals with masses of *m/z* 367, 489, and 258 were also detected and correspondingly assigned to structures **23**, **24** and **25** (Scheme 6). Taking this in consideration, we proposed that the mechanism by which the drug is released, probably initiates with GSH thiol addition to the

electrophilic imine carbon centre of **20** to form intermediate **21** present in the reaction mixture (*m/z* 912). DFT calculations performed on a simplified model,[13] suggest that thiol addition destabilizes the complex by increasing the electronic density on the B-atom bond, weakening the B-O bond of the aminophenol (Scheme 6, see also section 6.2 SI). This process leads to the opening of the 5-member ring and to the formation of intermediate **22** in which **Btz** (*m/z* of 367) is more solvent exposed and consequently more susceptible to hydrolysis. This corresponds to the less stable intermediate along the path, 15 kcal/mol above the reagents. This mechanism is also supported by the presence of compound **24** (*m/z* of 489), which probably derives from an intramolecular addition of the amino group on to the GSH amide and consequent release of a glycine residue. Hydrolysis of **24** forms the imine **25** that is also clearly present in the reaction mixture (*m/z* 258). The free energy balance calculated for the entire process indicates a fairly exergonic reaction with ΔG = –6 kcal/mol.



Scheme 6. ESI-MS analysis of the conjugation reaction between B-complex **20** and GSH.

In summary, here is presented for the first time a modular platform to construct cancer cell targeting drug conjugates, using BAs to promote the assembly of functional components featuring a cytotoxic drug (**Btz**), Peg chains and azide handles. The multivalent boronated core was assembled in a single step (up to 25 % isolated yield) and the azide component was effectively post-functionalized with folate targeting units *via* a SPAAC reaction. This molecular architecture afforded conjugates with high selectivity against folate positive MDA-MB-231 cancer cells and IC_{50} in the nanomolar the range. The boronated core was shown to be quite stable in human plasma (up to 60 h half-life), though reversible in the presence of GSH. The intracellular delivery of the cargo attached to the BA component was confirmed by confocal fluorescence microscopy. Based on mass spectrometry and DFT calculations, was proposed a reaction mechanism for the GSH induced hydrolysis of the B-complex that involves the GSH thiol addition to the electrophilic imine carbon. Considering the boronated core straightforward modularity, stability in human plasma and stimulus responsiveness, the availability of cytotoxic drugs that maybe simply borylated (e.g. SN38), this platform evidences unique properties to be used in the construction of multivalent therapeutic conjugates and as a protective group for BAs that blocks the undesired reactivity of this function.

Acknowledgements

The authors thank Fundação para a Ciência e a Tecnologia (FCT), Portugal (grants: SFRH/BD/94779/2013, PTDC/QEQ-QOR/1434/2014, UID/DTP/04138/2013, SAICTPAC/0019/2015 (iMed.ULisboa), UID/QUI/00100/2013 (LFV). Patent rights granted to Hovione SA PT109941.

Keywords: Boronic acids • Multivalent • Bortezomib • SMDC

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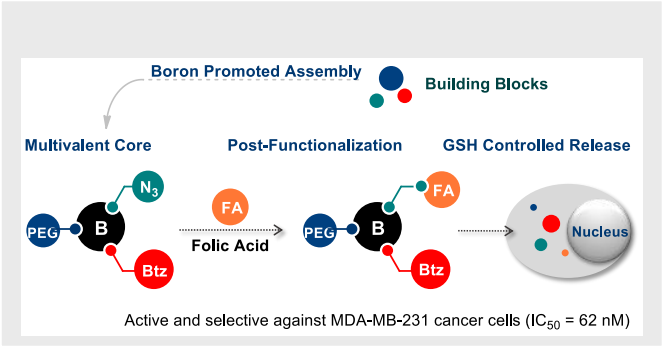

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Page No. – Page No.

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