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Benzoxaboroles as dynamic covalent receptors for bioconjugation and transport of nucleosides and related drugs: Proof of action in HeLa cells

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ABSTRACT

In this work we describe not previously explored binding studies on the reversible interaction of benzoxaborole with ligands of medical and pharmaceutical interest such as nucleosidic drugs gemcitabine and capecitabine, as well as the hydrophobic chemotherapeutic doxorubicin. We include functional derivatives of benzoxaborole such as a near infrared fluorescent boronolectine, Cy-Bx, The dynamic covalent interaction in physiological conditions was assessed by spectroscopic techniques yielding moderate to high binding affinities. The cytotoxic activity of the drugs upon conjugation to the boronolectins was evaluated revealing significant influence of the bioconjugation status on the cellular viability. The availability of the conjugate for cellular uptake and localization in the model cancer cell line HeLa was assessed by fluorescence imaging. Benzoxaborole and the fluorescent boronolectin Cy-Bx, proved to be versatile conjugation tools for 1,2 and 1,3-diol containing pharmacophores as well as bioisosteric forms such as 1,2-hydroxyamino, envisioning these small boronolectins as components in systems for drug release with tracking capability.

1. Introduction

Scientific interest in benzoxaborole, a boronic hemi-ester, was boosted in recent years because of its promising activity toward a variety of targets such as fungi, bacteria and parasites [1–3]. Since the work of Hall & col., we also know that benzoxaborole possess high water solubility, low pK_a (in comparison to phenylboronic acids) and enhanced affinity toward *cis*-diols motifs under physiological conditions, overcoming the limitations of phenylboronic acids as receptors for diol-containing molecules of relevant importance such as sugars [4–5]. This reactivity toward diols has been exploited with different purposes and applications: bioconjugation tags [6–7], sugar responsive materials [8–10], protein delivery [11], glycoprotein enrichment and immobilization [12] and molecular fluorescent probes for glycoproteins [13–15] as well as sugar sensing [16–17]. We have been focused on creating fluorescent probes for glycoconjugates in the lasts years, which led us to insight into the binding abilities of benzoxaborole and to expand our interest on this molecule and it potential application especially regarding the boronic-diol interaction as a tunable conjugation methodology. On this regard, we turn to study the recognition of nucleosides and related structures as target ligands for benzoxaborole due to their importance as active biomolecules. Comparatively to what is reported on saccharide recognition or bioconjugation strategies, the binding of benzoxaborole to nucleotides and nucleosides is much less studied and exploited. The motivation is to establish whether benzoxaborole could be a safe partner for bioconjugation of nucleoside-like hydrophilic drugs such as capecitabine and gemcitabine, facilitating their passive permeation. Since -F, -OH and -NH_2 are isosteric groups according to the Grimm's hydride displacement law [18], we expect that vicinal *cis*-fluor alcohol and *cis*–amino alcohol motifs, binds to the boron center in a similar manner that *cis*-diol groups do.

Therefore, another interesting compatible structure suitable for the interaction with benzoxaborole is the *cis*-amino-ol sugar like moiety contained in the chemical structure of the hydrophobic chemotherapeutic agent doxorubicin. This motif is also found in threonine, the aminoacid that interacts with Bortezomib within the catalytic site of the proteasome, evidenced by X-ray spectroscopy [19].

Nucleoside analogues are continuously investigated as therapeutic agents [20,21], whether alone or in conjugated systems. Conjugation of drugs is often studied as an alternative to overcome issues such as drug

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resistance and bioavailability. Few examples are found in scientific literature accounting for the use of the boronic-diol interaction as a bioconjugation strategy. For instance, phenylboronic acid interaction with the ribose like fragment of capecitabine has been exploited recently by Ma & col. to encapsulate this 1,2-*cis* diol containing drug together with an amphiphilic block copolymer aided by the formation of an imidoboronate interaction [22]. Also boronic acid functionalized polycarbonates were used to complex capecitabine into assembled nanoparticles with appropriate acidic response to release the drug [23]. Kim & col. designed a theranostic molecule containing the prodrug 5′-deoxy-5-fluorouridine anchored to a boronic acid residue, that release the active compound by interaction with H_2O_2 [24]. On the other hand, it was found that the dipeptydil boronic acid named Bortezomib, showing non growing activity of malignant cells by proteosome inhibition, sensitize the action of chemotherapeutic agents like gemcitabine and 5-fluorouracil [25,26]. When it comes to doxorubicin, we can find some examples describing its encapsulation within different types of matrices based on the crosslinking between boronic acid groups and *cis*-diols [27–30].

Regarding the superior properties of benzoxaborole as a receptor for *cis*-diols in physiological condition over phenylboronic acids, we describe the binding of benzoxaborole and related functional structures to relevant nucleosides and related drugs by means of simple spectroscopic methods. We also analyzed the viability of HeLa cells when this cell line was treated with the conjugates and demonstrate the non-perturbed cellular up-take and localization of the conjugated doxorubicin in live cells. Complementary, a fluorescent benzoxaborole derivative (**Cy-Bx**) developed in our lab emitting in the near infrared region is presented as well as a pro-drug yielding evidence on the internalization and localization of the drug by dual channel observation.

2. Experimental

2.1. General experimental procedures

Corrected fluorescence emission spectra were obtained on a Cary Eclipse spectrophotometer equipped with two Czerny-Turner monochromators and a 15 W Xenon pulse lamp (pulse width: 2–3 us, power: 60–75 kW). When temperature control was necessary, the cell was thermostated using the Thermal Application of the ADL software.

Fluorescence microscopy was performed with an Olympus Fluoview FV 1000 microscope with a UPLSAPO 60x 1.4NA oil immersion objective. Excitation and emission filters were as follows: excitation DAPI, 405 nm; emission DAPI, band pass (BP): 430–470 nm; Doxorubicin 485 nm; emission Probe, BP: 550 nm; **Cy-Bx** 637 nm; emission Probe, BP: 655–755 nm. We always used the sequential mode for image acquisition.

^{1}H NMR and ^{13}C NMR spectra were measured with a Varian Bruker AC-200, Bruker Fourier 300 and Bruker Avance II-500 spectrometers (UMYMFOR-CONICET-UBA). Mass spectra were measured with a Bruker Daltonik microTOF mass spectrometer (ESI^+, ESI^-).

2.2. Binding experiments in vitro

Solutions in the range 0.05–0.1 mM of boronolectins in buffer PBS 0.1 M, pH 7.4 were used to prepare stock solutions for titration with the different analytes. By mixing the boronolectin solution with the analyte solution together, a range of analyte concentrations (0–200 mM for monosaccharides, 0–50 eq for nucleos(t)ides and drugs) was obtained. After 10 min of incubation, the fluorescence emission corresponding to the receptor was measured (λ_{exc} 260 nm) in 10 × 1.5 mm quartz cuvettes. Plots of $1/\Delta F$ vs 1/[analyte], where $\Delta F = F–F^0$, were fitted to compute the apparent binding constant, K_a (M^{-1}), as the ratio between the intercept and the slope.

2.3. Cell culture and MTT viability assay

The MTT assay involves the conversion of the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to an insoluble purple formazan by the action of mitochondrial reductase. This method was used to evaluate cytotoxicity of the drug-conjugate with benzoxaborole and free drug. Briefly, 5×10^3 HeLa cells were seeded into a 96-well plate and subsequently incubated in a humidified (> 95%) incubator at 37 °C with 5% CO_2 overnight with Dulbecco's Modified Eagle's Medium (D-MEM), 10% SFB and 50 U/ml penicillin, and 50 mg/ml streptomycin. Next day, D-MEM was exchanged with 200 µL fresh culture media including free drug and benzoxaborole conjugated drug at 40, 22 and 60 µM for gemcitabine, capecitabine and doxorubicin, respectively. Benzoxaborole was also added into a series of wells to evaluate the cytocompatibility. Three wells with 200 µL of culture medium were used as control and three wells containing cells without drug were used as reference since it has the maximum cell viability. All treatments were incubated for 24 h. After incubation, D-MEM with drugs was removed and washed twice with PBS. 200 µL per well of MTT (5 mg/mL) were added and incubated during 2 h at 37 °C. Then, MTT solution was removed and the purple formazan dye crystals were solubilized by the addition of 200 µL of DMSO. The absorbance was recorded at 570 nm in a plate reader PolarStar BMG, LABTECH.

2.4. Statistical analysis

The data obtained from the MTT assay in HeLa cells are expressed as the mean ± standard deviation (n = 4). The statistical significance was analyzed by means of a 2- way ANOVA with interactions (benzoxaborole–drug) in a completely randomized design. The assumptions of normality and homoscedasticity were studied analytically by the Shapiro-Wilks and the Levene tests, respectively. Tukey's test was used for post hoc comparisons. Results were considered significant at $p < 0.05$. Means with the same letter represent not significative differences. All statistical analyses were performed using the statistical program Infostat (Universidad de Córdoba, Córdoba, Argentina).

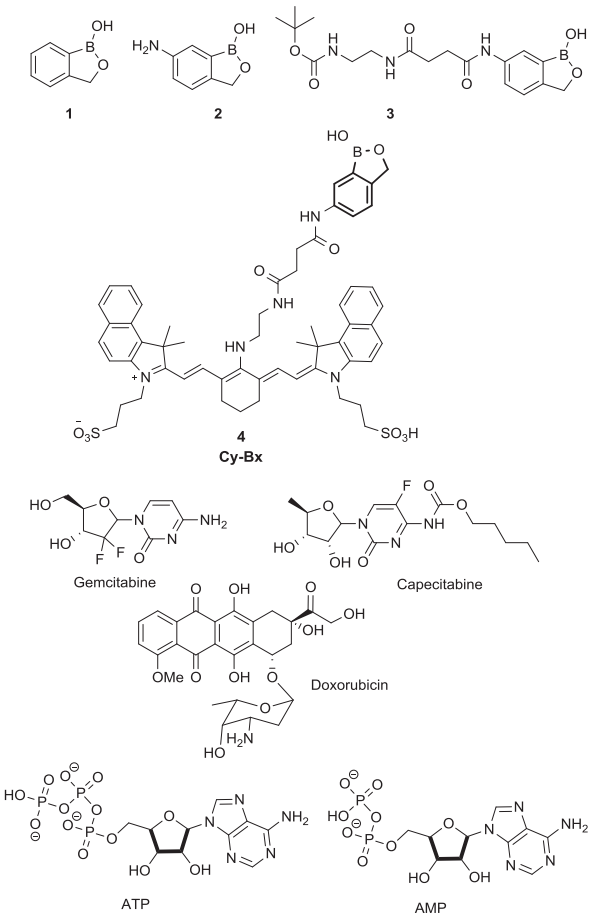
2.5. Imaging of doxorubicin and *Cy-Bx* localization in HeLa cells.

HeLa cells were maintained in D-MEM (Dulbecco's Modified Eagle Medium) supplemented) and 10% FBS, 50 U/mL penicillin and 50 µg/mL streptomycin (Gibco). The cells were harvested when 80–100% confluent with TrypLE™(Gibco, Denmark), Select 2X, 1 mM EDTA collected, and centrifuged at 200*g* for 5 min. Then, cells were seeded on 12 mm cover slips previously coated with poli-D-lysine and collagen. Culture media was removed and cells were gently washed with PBS and incubated at 37 °C for 30 min in modified Tyrode's buffer containing 50 µM of DOXO- and DOXO/**Cy-Bx** conjugate in a 10:1 ratio, at different concentrations. After incubation cells were washed three times before fixation with PFA 4% in PBS and permeabilized with Triton X-100 0.025% in PBS. Then, the cells were incubated with DAPI 1X in PBS for 10 min at room temperature. After that, cells were washed three times with PBS and mounted with Mowiol (Sigma-Aldrich) Images were taken with an epifluorescence microscope (Olympus FV 1000, objective oil 60X, NA = 1.42) and analyzed employing FIJI ImageJ software.

3. Results and discussion

3.1. Binding of boronolectins to nucleosidic analytes

Synthetic receptors derived from the boronic acid family are named as *boronolectins*. The chemical structure of the studied boronolectins and analytes are shown in Scheme 1. Benzoxaborole (BX, **1**), aminobenzoxaborole (ABX, **2**), the linkered derivative (BXSEDA, **3**) and the NIR-fluorescent benzoxaborole (Cy-Bx, **4**) were synthesized by simple methodologies (see ESI).



Scheme 1. Chemical structures of benzoxaborole (1), functional boronolectins (2, 3), fluorescent boronolectin (4) and model drugs and nucleotides evaluated in this study.

Compounds **2** and **3** are valuable functional analogues to perform orthogonal derivatization of the benzoxaborole unit with fragments of interest such as fluorophores, polymer precursors, surfaces, ligands for direct site conjugation such as folic acid, among others to generate targeted cytotoxic compounds. The benzoxaborole receptors showed excellent water solubility and dissolved clearly at the range of concentration used (0.05–0.10 mM) in buffer PBS 0.1 M pH 7.4 without the need of using an organic co-solvent.

We first study the binding of model analytes by direct monitoring spectral changes of the receptors, a methodology that allow us to avoid an indicator displacement assay which is more time consuming and needs complicated data analysis, or other spectroscopic methods like NMR which require larger amounts of samples. Especially we focused on the use of fluorescence detection due to the gain in sensitivity. At a concentration range of 0.05–0.10 mM, the three boronolectines have significant fluorescence emission in the UV region 280–380 nm when excited at 260 nm (Fig. S1). The changes of the emission maximum with pH were determined to compute the pK_a values yielding 7.3, 7.4 and 6.9 for **1**, **2** and **3**, respectively, matching conveniently the physiological range (Fig. S3). The binding ability toward fructose, glucose, adenosin monophosphate (AMP) and adenosin triphosphate (ATP) as representative ligands, was monitored by the change of emission for each receptor by direct titration experiments. The binding isotherms for the receptors **1**, **2** and **3** are shown in Fig. S4. A decrease of the fluorescence emission was observed along with increasing amounts of the analytes allowing for the calculation of the corresponding apparent binding constants assuming 1:1 binding model (Fig. S5, Table S1). The quenched emission upon addition of the guests correlates with the changes observed for the emission of the receptors when moving from acid to basic pH values: when the tetrahedral ionized state of the boronate predominates, decrease in the fluorescence emission is observed.

A simple qualitative analysis from graphical comparison reflects the difference in affinity toward monosaccharides on one side and toward nucleotides on the other. The computed values are in the expected range for monosaccharides compared to binding constants obtained by other methods. [4] The results are consistent with other boronic acids reported.

Within the sugar group the higher affinity is, as expected, toward fructose because it predominates in the furanose conformation in solution (22% for fructose against 1% for glucose). The different binding properties are related to the different dihedral angles of the diols. Between receptors, the highest K_{ap} value for the saccharides was computed for receptor **3.** Boronolectin **3** has also the lower p*K*a (6.9) resulting in more stable cyclic boronates with monosaccharides in physiological condition (pH 7.4). It is expected that with lower p*K*a of the boronic acid receptor, higher affinity is reached toward *cis*-diols because more tetrahedric boron is available to form the cyclic boronate. However, this is not a fixed rule, as can be seen with values computed for receptor **3** an its binding toward ATP and AMP, reflecting that other factors, for instance steric and electronic issues, affect the interplay between receptor and ligands.

Nevertheless, the substitution pattern on the aniline nitrogen atom reflects differences in the fluorescence properties of these compounds (Fig. S1) but does not affect the molecular recognition of these receptors for different analytes in a significant way. All receptors bind to nucleotides tightly given that the ribose ring is fixed in the furanose form by the covalent bond to the nitrogenated base.

Within the nucleoside group, benzoxaborole **1** binds to adenosine, cytidine and uridine with similar apparent binding constant: adenine 8000 M^{-1}, cytidine 7777 M^{-1} and uridine 8000 M^{-1}, while we computed a higher value for the binding to guanosine (17,500 M^{-1}) (see Fig. 1). It has to be accounted that base pairing through π-π and hydrogen bond interactions are additive and cooperative and may introduce variations in the value of the K_{ap}. The higher tendency of guanosine to establish π-π stacking was previously described [31].

Given the high affinity observed by benzoxaborole toward model nucleo(s)tides we turn our interest in the study of the bin- ding of **1** with nucleosidic model drugs, gemcitabine and capecitabine.

The affinity of benzoxaborole toward the drugs in PBS buffer pH 7.4 was at the level of the tested nucleotides or even higher. The computed values were 8000 M^{-1} for gemcitabine, 11,500 M^{-1} for capecitabine and 5333 M^{-1} for doxorubicin.

The 1:1 complexes between benzoxaborole and hydrophilic drugs gemcitabine and capecitabine, in water solution, were detected by ESI-MS (Fig. S7). Further evidence of the interaction was achieved by

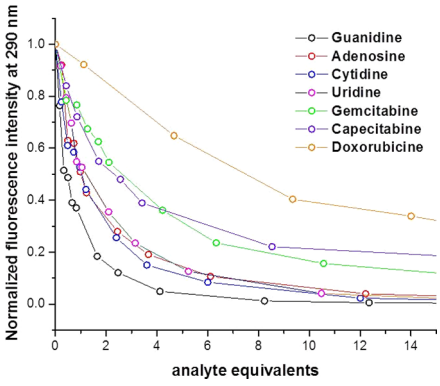


Fig. 1. Change in the emission of benzoxaborole 1 (5×10^{-5} M) upon binding of different model nucleosides, nucleosidic drugs gemcitabine and capecitabine, and the hydrophobic drug doxorubicin, in buffer PBS 0.1 m, pH 7.4. λ_{exc} = 250 nm.

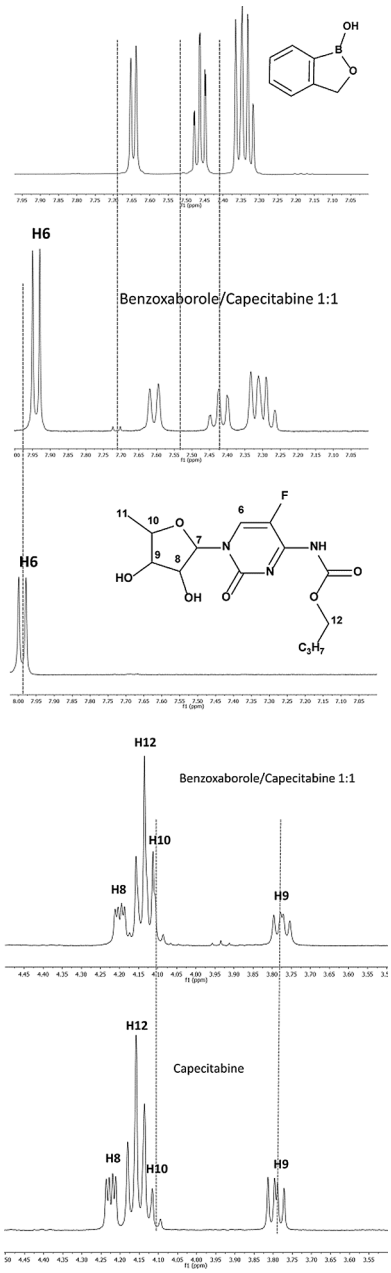


Fig. 2. ^{1}H NMR spectra of 0.04 M benzoxaborole, 0.04 M capecitabine and the 1:1 mixture of both in D_2O.

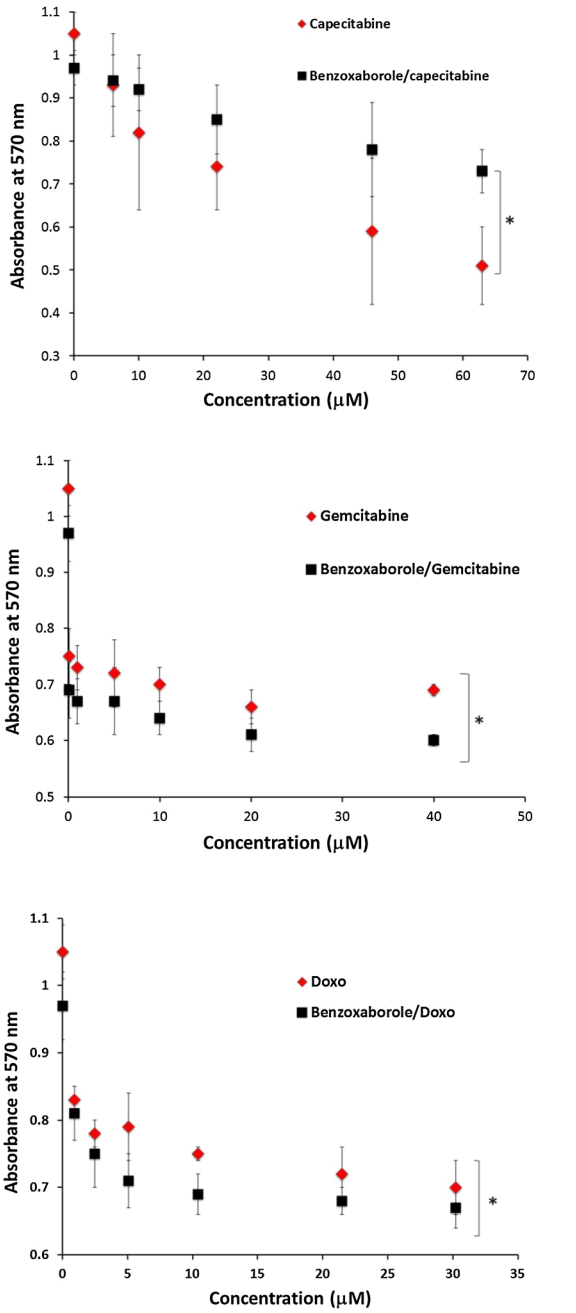


Fig.3. Cytotoxicity of the different Benzoxaborole/drug conjugates compared to free drug. Cell proliferation of HeLa cells after incubation with complexes vs. free drug was determined by MTT assay. Graph shows mean ± SD absorbance at 570 nm reflecting cell viability, performed in quadruplicate. * denotes a statistically significant difference in toxicity between free drug compared to the conjugates in all cases ($0.01 < p < 0.05$).

inspection of changes of NMR signals of the benzoxaborole and the drugs whether alone or in the conjugate state. As illustrated in Fig. 2, a significant peak broadening in the aromatic region of the ^{1}H NMR spectrum was observed when capecitabine was added to the benzoxaborole solution in D_2O to form the 1:1 complex. Moreover, the signals assigned to H8 and H9 of the ribose unit were also broadened in presence of receptor **1**.

In the case of the complex between benzoxaborole and gemcitabine, we expected to find evidence of binding through the *cis*-fluor-alcohol motif. A comparison of the ^{19}F NMR spectra of gemcitabine with or without benzoxaborole, showed neither shift nor broadening or other kind of change adjudicable to the interaction (Fig. S10). However, it was evident from comparative analysis of the ^{1}H NMR spectra, that the binding was taking place, not only by broadening and shifting of the aromatic signals but also from the changes observed in the signals of H9, H10 and H11 of the ribose unit, indicating that the recognition event is established through the 1,3-diol motif present in gemcitabine, rende- ring a six membered cyclic boronate (Fig. S8). Exploring the binding between benzoxaborole and doxorubicine by NMR in D_2O was precluded by drug insolubility in water at the mM level required for the experiment. Nevertheless, slightly changes could be observed for benzoxaborole and doxorubicine signals by ^{1}H NMR when the 1:1 complex was formed in DMSO-d_6 (see Fig. S9).

3.2. Cellular toxicity of the conjugates in HeLa cells

With this evidence of complex formation between **1** and the drugs, we studied the influence of the complexation with benzoxaborole in the

cytotoxic activity of the drugs through the cell viability based tetrazolium assay (MTT) with HeLa cells. Cells were treated with different concentrations of conjugates [benzoxaborole: drug] and compared to treatment with free drug. From Fig. 3 it can be seen that the cytotoxicity of the conjugates exhibits a dose-dependent activity similar to that of free drugs. The presence of benzoxaborole as a conjugation partner rendered non-predictable influence on the cytotoxic activity of the drugs in the model cancer cell line tested. In the case of gemcitabine and doxorubicin, the complexed drugs showed slightly higher cytotoxic behavior than the free drug while the opposite was found with the capecitabine conjugates.

Since benzoxaborole shows no toxic effect by itself according to the control experiment (Fig. S12), the enhancement in cytotoxicity could be ascribed to a better permeation of the drugs when conjugated to the boronic hemiester, which impacts in their bioavailability. It is known that passive membrane diffusion is not themain transport pathway for ribonucleosides and that nucleoside drugs enters cells via membrane-bound nucleoside transporters [32]. Smith & col. have also shown that boronic acids facilitate the transport of nucleosides and other ligands through liquid membranes [33]. Our results are in accordance with these observations in artificial membranes and moreover, it also works for the *cis*-amino alcohol motif found in doxorubicin. In the case of capecitabine, a slower or less efficient passive transport could be occurring in comparison with the other conjugates and it should be also taken into consideration that as a pro-drug and it has to suffer five enzymatic modifications within the cell to become active. [34]. The decrease of toxicity for the conjugated drug might be a result of less availability of the active compound due to the binding situation to the benzoxaborole, retarding the activity.

Nevertheless, the conjugate proved to be cytotoxic at acceptable level, and it is not to be considered a discouraging result, since retarding the drug activity due to bioconjugation could be an advantage when it comes, for example, to targeted theranostics.

3.3. Cellular uptake and localization of conjugated doxorubicine

The intrinsic fluorescence of doxorubicin is a convenient tool to compare the uptake and localization of the drug inside the cells (Fig. 4). Fluorescence microscopy reveals that after 4 hs of incubation with doxorubicin and benzoxaborole conjugated doxorubicin, the fluorescence signal in the green channel (Doxo) is mainly located in the nuclei, as expected. Conjugation of the drug does not compromise the cellular uptake and appropriate localization of the drug.

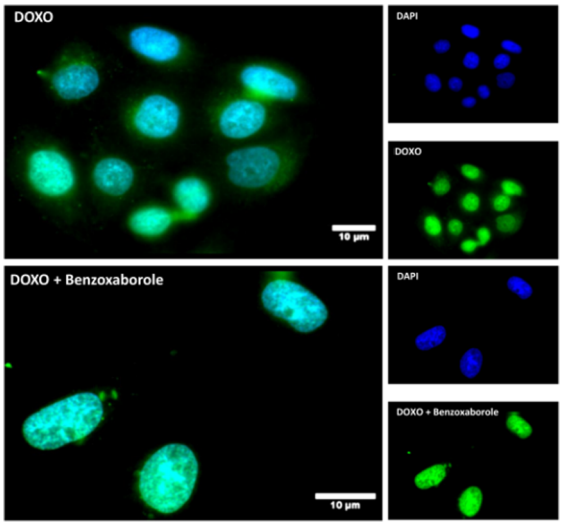


Fig. 4. Fluorescece microscopy images of Hela cells incubated with free (upper image) and benzoxaborole conjugated doxorubicin (lower image) for 4 hs. Scale bar 10 µm.

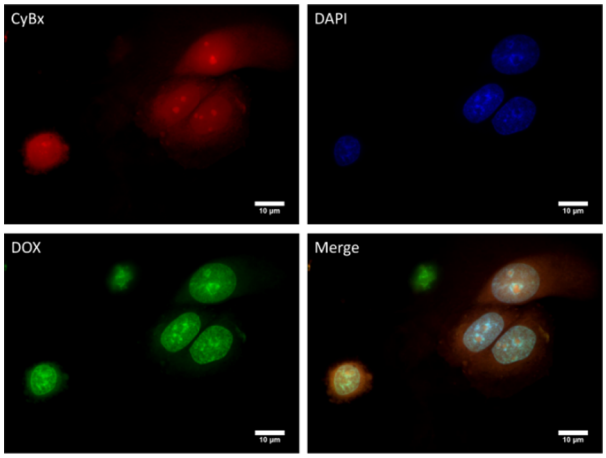


Fig. 5. Fluorescence microscopy images of HeLa cells incubi- ted with the conjugate CyBx-Doxo for 4 hs. Red channel (upper image left), blue channel (upper image right), green channel (lower image left) and merge. Scale bar 10 µm (lower panel).

More interesting results were obtained when we next explored evidence of conjugation between our previously reported near infrared boronolectin, **Cy-Bx**, which has a benzoxaborole unit linked to the fluorophore tricarbocyanine. [14]

In solution, the binding of **Cy-Bx** with drugs was studied qualitatively by titration experiments monitoring the quenching of the emission at 780 nm (Fig. S11) yielding almost a complete quenched sate for doxorubicin. The cytotoxic activity of the **Cy-Bx**/DOXO complex correlates with free Doxo upon increasing concentration of the drug and the tight conjugation state observed in vitro imparts less toxicity to the cells (Fig. S13).

Observations achieved by fluorescence microscopy imaging (Fig. 5, lower pannel) allow us to infer that the complexation situation did not affect the permeation of **Cy-Bx** inside the nucleus, with a high emissive signal of the free NIR fluorophore, a fact not previously observed when this dye and similar fluorescent boronolectins were used to label glycoconjugates within cells [13,14].

Moreover, **Cy-Bx** became highly emissive within dots that corresponds to nucleolus, the largest structure in the nucleus, very rich in ribosomal RNA. The binding of benzoxaborole derivatives of pharmacological interest to the terminal nucleotide unit of RNA has been previously studied supporting this observation [35].

In summary, we present binding studies at physiological pH in absence of co-solvent, between benzoxaboroles and relevant therapeutic agents, as a prodrug combination not previously explored. The above described bioconjugation methodology has great potentiality for intracellular delivery relying in a dynamic covalent bond that is stable enough at pH 7.4 to influence the passive permeation of nucleosidic drugs through the cell membrane. Moreover, conveniently functionalized benzoboroxole derivatives such as receptors **2** and **3** does not alter the dynamic covalent binding and offer the opportunity to be combined with appropriate fragments for tumor site-directing targeting (folic acid, RGD peptide, organelle-specific ligand) and with imaging agents.

4. Conclusion

We show that the very well known interaction based on boronic acids and *cis*-diols can be expanded to structurally related motifs such as *cis*-hydroxy amino and 1,3-dihydroxy configurations with high affinity toward the boron center. As it is known, this type of bioconjugation can be modulated by external stimuli such pH, active oxygen species or competitive analytes. Finally, a NIR fluorescent boronolectin was applied in conjugation to doxorubicin as a proof of principle of potential

applications of this molecule as a receptor-reporter tool in dynamic bioconjugation. We expect our report contributes in the search for innovative bioconjugation strategies of medicinal agents and in the development of novel theranostic systems especially facing the problem of selectivity toward abnormal cells and drug resistance. Research of this conjugation strategy in macromolecular systems for multivalent conjugation is currently under way in our group.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103059.

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