B and H-NMR Evaluation of Chemical Interactions Between Two Polyphenols; Epigallocatechingallate and Quercetin with Bortezomib

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Bortezomib (Velcade) is a boronic acid dipeptide, proteasome inhibitor drug that its antimyeloma activity in treatment of patients with multiple myeloma (MM) has been approved by FDA. Studies show that consumption of daily diet rich in polyphenols affect Bortezomib's bioavailability and reduce its antitumor activity. In the present study we made an attempt to investigate the interactions between Bortezomib and some common polyphenols to examine whether these polyphenols interact with the drug. For this purpose, interactions between four dietary polyphenols including: Emodin, Rhein, Epigallocatechingallate (EGCG) and Quercetin with methyl boronic acid, Bortezomib's substitute, were studied using 1H and 11B- NMR spectroscopy. The results suggest that the vicinal diols in the structure of the two polyphenols (EGCG and Quercetin) interact with the boronic acid moiety of Bortezomib and convert the active triangular boronic acid to an inactive tetrahedral boronate through direct chemical interaction, and this conversion abolishes the antimyeloma activity of Bortezomib. In conclusion, our study suggests that the consumption of dietary sources enriched in EGCG and Quercetin during treatment with Bortezomib in MM patients should be considered.

Key words: Epigallocatechingallate, polyphenol, Quercetin, Bortezomib, chemical interactions.

Bortezomib, a boronic acid dipeptide, is a novel food and drug administration (FDA)-approved proteasome inhibitor drug that its antimyeloma activity has been shown in preclinical and phase 1 studies (Orlowski et al, 2002) and it is shown to be active in patients with relapsed multiple myeloma that are refractory to conventional chemotherapy (Richardson et al., 2003). In a variety of in vitro and in vivo studies Bortezomib has shown to have antitumor activity when administrated either alone (LeBlanc et al., 2002; Frankel et al, 2000) or in combination with other chemotherapeutic agents (Pink et al., 2002; Teicher et al., 1999) or radiation (Russo et al, 2001; Pervan, 2001). Inhibition of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is considered as the main mechanism behind the action of Bortezomib (Mitsiades et al., 2002; Hideshima, 2003). Studies also reported that Bortezomib is also involved in down regulation of several apoptosis inhibitors, adhesion molecules expressed in myeloma cells and bone marrow stromal cells as well as induction of caspase-dependent apoptosis, antiangiogenic activity and reducing the release of intracellular reactive oxygen species (ROS) (Mitsiades et al, 2002; Cusack et al, 2001).

Polyphenols- previously known as vegetable tannins- are plant secondary metabolites...
that have the characteristic of having several phenols- also known as carbolic acid- in their structure. Phenols are formed by attaching a hydroxyl (-OH) group to an aromatic benzene (phenyl) ring (Quideau et al., 2011). Because of their reducing characteristic, polyphenols have antioxidant capacity and together with other compounds such as carotenoids and Vitamins E and C, can protect the body tissues against oxidative stress (Scalbert & Williamson, 2000). Although many studies have previously suggested the prevention of various diseases through consumption of polyphenol-rich foods (Weber et al., 2003; Cabrera et al., 2006), new line of studies have brought drug-food and drug-herb interactions into focus (Chavez et al., 2006). Among their components, polyphenols have been confirmed to antagonize the antiproliferative effects of some antitumor drugs. A pervious study showed that green tea polyphenol; epigallocatechin-3-gallate (EGCG) interacts with antitumor drug Sunitinib and diminishes the bioavailability of Sunitinib in cancer patients (Ge et al., 2011). Other observations showed that EGCG acts as a multipotent chemopreventive and anticancer agent in several animal models, including leukemia, lung, prostate, colon, and breast cancer (Yang et al., 2006; Kurahashi et al., 2008; Khan & Mukhtar, 2007; Leong et al., 2008). Antagonistic effects of vitamin C (Dai et al., 2004), Tiron (Hideshima et al., 2005) and Quercetin (Chauhan et al., 2008) on Bortezomib have also been reported. Many in vitro cell-based and in vivo studies have supported the potential chemical interactions of dietary polyphenols with Bortezomib based on their structures (Kim et al., 2009; Ogawa et al., 2008).

In the present study, to investigate the potential interactions between polyphenols and Bortezomib, four pure dietary polyphenols including Emodin (6-methyl-1,3,8-trihydroxyanthraquinone), Rhein (4,5-dihydroxy-9,10-dioxoanthracene-2-carboxylic acid), Epigallocatechingallate ([(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate ) and Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) were selected and the possible chemical changes in the structure of Bortezomib in the presence of these compounds were analyzed using 1H-NMR and 11B-NMR spectroscopy.

**MATERIALS AND METHODS**

**Reagents**

Four dietary polyphenols including; Emodin (6-methyl-1,3,8-trihydroxyanthraquinone), molecular formula: C_{15}H_{10}O_{5}, molecular weight: 270.24 g/mol, Rhein (4,5-dihydroxy-9,10-dioxoanthracene-2-carboxylic acid), molecular formula: C_{15}H_{8}O_{6}, molecular weight: 284.22 g/mol, Epigallocatechingallate ([(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate), molecular formula: C_{22}H_{18}O_{11}, molecular weight: 458.4 g/mol and Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one), molecular formula: C_{15}H_{10}O_{7}, molecular weight: 302.236 g/mol and also Methyl boronic acid powder (5g) were purchased from Sigma-Aldrich (St Louis, MO, USA). Bortezomib (Velcade) powder in a 25mg vial was purchased from Selleckchem (Houston, TX). NMR solvents including deuterated water D_{2}O, saline solution (0.9% NaCl in 90% H_{2}O and 10% D_{2}O), deuterated

![Fig. 1. Chemical structures of A. Emodin, B. Rhein, C. EGCG, D. Quercetin. Vicinal diols can be seen in the structure of the last two](image-url)
methanol CD$_3$OD, deuterated DMSO (CD$_3$)$_2$SO, and deuterated acetonitrile CD$_2$CN plus 20% D$_2$O and a drop of deuterated acetonitrile CD$_2$CN added to it, were all supplied by the BMRC NMR facility (Sheffield Hallam University).

**NMR instrumentation**

An Advance III 400 MHz NMR spectrometer (Bruker) fitted with a BBO probe was used for $^1$H-NMR and $^{11}$B-NMR spectroscopy. The spectra that were obtained using $^1$H-NMR spectroscopy used a standard zg30 program for acetonitrile, methanol and DMSO samples and a water suppression program called zgcppr for D$_2$O and saline solution samples with 16 (sometimes 128) scans. For $^{11}$B-NMR spectroscopy the program used was the standard zg program with 128 scans. The NMR tubes included regular glass tubes which were supplied by Fisher Scientific and quartz tubes (Wilmad) (which do not contain boron in them and are preferred to normal tubes where looking for boron nuclei is desired) were supplied by Sigma-Aldrich. An ultrasonic water bath (Sonicor Instrument Corporation, SC-200-22) was used for heating the samples.

**Sample preparation**

In order to find the most suitable NMR solvent(s), the polyphenols (Emodin, Rhein, EGCG, Quercetin), Bortezomib and its substitute methyl boronic acid, were dissolved in five different solvents including: deuterated water(D$_2$O), Saline solution, Methanol, Acetonitrile plus 20% D$_2$O and Dimethyl sulfoxide (DMSO). Also their maximum dissolving capacity was recorded. To dissolve the polyphenols in D$_2$O, saline solution, and acetonitrile plus 20% D$_2$O, the samples were heated at 50°C for an hour. All samples were shaken vigorously for a few seconds and then they were left to stand for a few minutes. Afterwards the NMR spectra for both boron and hydrogen nuclei were recorded. The aim was to find out which of the four different polyphenols have the ability to change the chemical shifts of methyl boronic acid in both/either boron and hydrogen NMR spectra. In other words to monitor a change in the peaks that led to a new spectra, that resembled neither the methyl boronic acid spectra nor the polyphenol spectra alone.

All samples were kept in the fridge (4°C) while they were in NMR tubes and wrapped in aluminum foil, before they were used again to record further spectra later over the following days. Experiments which were indicative of interactions between methyl boronic acid and polyphenols in the previous section were repeated this time with the drug Bortezomib instead of methyl boronic acid. According to the results obtained from the previous section, only the two polyphenols: Quercetin and EGCG were selected to be examined by $^1$H-NMR and $^{11}$B-NMR spectroscopy for their interactions with Bortezomib.

All samples were weighed and the mixtures were adjusted at 40 folds excess of Quercetin compared to the drug (0.01M Bortezomib solution + 0.4M Quercetin solution were mixed up) and about 10 folds excess of EGCG. All samples were heated for an hour using the ultrasonic water bath to make sure they are dissolved. $^1$H and $^{11}$B NMR spectra were recorded.

**RESULTS**

**Finding the best solvent(s)**

According to the results obtained from the solubility tests (data not shown), two polyphenols Emodin and Rhein either did not dissolved in any of the solvents or produced no $^1$H-NMR or $^{11}$B-NMR spectra and therefore were excluded from our study.

OH (hydroxyl) protons are not always observed in NMR solvents for various reasons. A large peak arising from water prevented hydrogen peaks from being observed in the solvents D$_2$O and saline solution. The quartz NMR tubes were
found out to be not very effective, as the boron spectra had a noisy baseline using either regular or quartz tubes. Deuterated methanol, acetonitrile and DMSO were found out to be more suitable compared with the two other solvents as there was a better chance to see the expected peaks and to get an improved spectra with higher resolution while using them as solvents. Therefore, for the second part of the work only these three NMR solvents were used. It was also found out that although the drug Bortezomib was soluble in all five solvents, and the proton peaks were detectable using any of the three suitable solvents, the boron peak could only be detected while deuterated methanol was used as the solvent (reason unknown).

**Methyl boronic acid chemical Shifts and Interactions**

To confirm the inhibitory effect of polyphenols on Bortezomib, we studied the reaction of polyphenols with methyl boronic acid and Bortezomib by getting 1H and 11B NMR spectra. We began our study by looking at the reactions between Quercetin and methyl boronic acid in the solvent DMSO. 1H NMR spectra of methyl boronic acid, Quercetin and a mixture of them both are shown in figures 2 and 3,

![Fig. 2. 1H-NMR spectra of methyl boronic acid (solvent=DMSO). δ= 0 ppm: methyl group, δ= 2.5, 3.3 ppm: hydrogen atoms of DMSO and residual water δ= 7.3 ppm: 2 hydroxyl groups](image1)

![Fig. 3. (a) 1H-NMR spectra of the Quercetin and (b) 1H-NMR spectra of the mixture of Quercetin and methyl boronic acid (solvent = DMSO)](image2)

![Fig. 4. a) 1H-NMR spectra of EGCG and b) 1H-NMR spectra of EGCG and methyl boronic acid (solvent=DMSO)](image3)
Fig. 5. (a) $^{11}$B-NMR spectra of methyl boronic acid, the single peak at 32 ppm represents the boron atom. (b) $^{11}$B-NMR spectra of the mixture including both methyl boronic acid and Quercetin (solvent = DMSO)

Fig. 6. (a) $^1$H-NMR spectra of Bortezomib, (b) $^1$H-NMR spectra of Bortezomib and Quercetin (solvent=DMSO)

Fig. 7. (a) $^{11}$B- NMR spectra of Bortezomib, (b) $^{11}$B- NMR spectra of Bortezomib and Quercetin (solvent = MeOD)
respective.

Peaks from both unreacted Quercetin and unreacted methyl boronic acid are detectable plus a new peak that can be seen at about 11 ppm.

In the next effort, we investigated the interactions between methyl boronic acid and EGCG. The peak about at about 11 ppm is also detectable in the mixture and the results are shown in figure 4.

To obtain clear evidence on the direct chemical interaction between polyphenols (Quercetin, EGCG) and boronic acid moiety of Bortezomib, the 11B NMR spectrum of methyl boronic acid was recorded in the absence and presence of the polyphenols, respectively. The large peak at 32 ppm represents the unreacted and trigonal structure (sp2) of boronic acid and the small peak at 20 ppm is a characteristic of the tetrahedral structure (sp3) of boronate in the presence of Quercetin (Figure 5). Adding EGCG to methyl boronic acid also resulted in the appearance of a new peak at about 15 ppm (figure 6).

The boron peak at 32 ppm represents methyl boronic acid and the other peak at about 14.93 ppm shows the boron atom in the new complex (solvent=DMSO). The curvy baseline in this figure was not resolved with further baseline corrections. The reason for it is unknown but it is possible that small amounts of the both methyl boronic acid and EGCG have resulted in gaining

![Fig. 8](image1.png)  
![Fig. 9](image2.png)
Bortezomib Chemical Shifts and Interactions

In the next effort, we investigated the $^1$H and $^11$B chemical shifts of the drug Bortezomib in the absence and presence of polyphenols. The $^1$H-NMR spectrum of Bortezomib-Quercetin mixture in DMSO represented a new peak at 11 ppm (figure 7) and $^11$B-NMR spectrum exhibited peaks at 13.98 ppm and 18.53 ppm for Bortezomib and the new complex (Bortezomib-Quercetin), respectively (figure 8).

In $^1$B-NMR spectrum, the complex (18.53 ppm) is showing the characteristic of the tetrahedral structure (sp3) of boronate in the presence of Quercetin in an appropriate region of the spectrum.

We also examined the interactions between Bortezomib and Quercetin in Metanol as solvent and the peaks at about 12 ppm is detectable (figure 9).

Ultimately, we investigated the interactions between EGCG and Bortezomib by NMR spectra, and the new peaks were not involved in either $^11$B or $^1$H-NMR spectra (probably due to the low concentrations of EGCG) (Figure 10).

**DISCUSSION**

Investigating the mechanistic understanding of the effects on Bortezomib, we hypothesized that the vicinal diols in the polyphenols interact with the boronic acid moiety of Bortezomib and convert the active triangular structure of boronic acid in Bortezomib to an inactive tetrahedral boronate through direct chemical interaction. The equilibrium of this conversion is controlled by the structure and concentration of polyphenols, and this conversion
abolishes the antitymoma activity of Bortezomib. We explored this hypothesis on the drug–drug interaction of Bortezomib with polyphenols using 1H and 11B NMR spectroscopy (figure 11).

The 26S proteasome is a 2.5MDa protein degrading machine built from approximately 31 different subunits. It contains two 19S regulatory complexes and a barrel-shaped complex (the 20S core complex) which is considered as the core catalytic part of the proteasome and is made up of two α and two β rings (Voges, 1999). We assume that boronate moiety of the drug reacts with Thr1 in the active-site of β5 subunit (one of the seven β subunits that form a β ring). Therefore, a structural change in this part may negatively affect the activity of 20S core part of the proteasome (figure 12).

A similar study discovered that tumor cell death induced by Bortezomib can be prevented effectively using various green tea components, in particular EGCG in vitro and in vivo, not only when they are used to treat MM cells but also glioblastoma cells as well (Golden, et al, 2009).

Another study by Kim et al (2009) also revealed that flavonoids such as Fisetin and Myricetin that have the vicinal diols in their structure were able to antagonize Bortezomib while other flavonoids without this particular structure such as Kaempferol and Molin were not (Kim, et al, 2009).

To summarize, we report here interactions between polyphenols and Bortezomib in methanol and DMSO. We found out that Quercetin had the ability to make a chemical modification in the structure of both methyl boronic acid and Bortezomib and create a tetrahedric complex. EGCG had no evidence for making the tetrahedral structure while it was used to antagonize Bortezomib, but the reason for that could be only small amounts of it being available. In addition, the two other polyphenols (Emodin and Rhein) were found out to be ineffective in structural modification and reaction with boronic acid and this ineffectiveness is probably due to the position of the hydroxyl groups on their aromatic rings known as vicinal diols, which are likely to react with the hydroxyl groups in the boronic acid moiety of the Bortezomib by forming an inactive and stable tetrahedral boronate adduct that mimics the tetrahedral intermediate that is made during substrate hydrolysis and this prevents Bortezomib from inhibiting the proteasome.

REFERENCES


