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Short communication

Ames positive boronic acids are not all eukaryotic genotoxins

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ABSTRACT

Boronic acids and their derivatives have been exploited for their pharmacological activity and their utility as intermediates in the synthesis of novel non-boron containing compounds. A recent study reported that boronic acids are bacterial mutagens. Here, results are reported from the testing of nine boronic acids using the pan-mechanistic eukaryotic GADD45a genotoxicity assays, BlueScreen HC and GreenScreen HC. Positive results were produced for one compound in GreenScreen and four compounds in BlueScreen. Only negative results were produced when tested with S9 metabolic activation. These data suggest that there is not a general genotoxic liability in eukaryotes, within this chemical domain. Furthermore, they are not potent eukaryotic genotoxins: positive results were produced only at concentrations between 1 mM and 10 mM. Their presence as low concentration contaminants or impurities would be unlikely to produce misleading positive results for a test material.

1. Introduction

Boronic acids are of interest in safety assessment because they are used as intermediates in the synthesis of pharmacologically active molecules. They may therefore be present as contaminants or impurities in compounds taken forward into development. O'Donovan et al. [1] reported the first investigation of genotoxicity in these compounds using the Ames bacterial reverse mutation assay. They found that 12 of 13 compounds tested produced positive results. All but one of these 12 were positive in TA100 and or WP2uvrA (pKM101) without S9 metabolic activation. The exception was also positive in TA1537. There was no evidence for adduct formation in these studies, but attention was drawn to the reactivity of these molecules with carbohydrate moieties found in ribonucleosides. Since these moieties are not restricted to prokaryotes, it is plausible that such indirect effects might also produce positive results in eukaryotic genotoxicity assays. Whilst there have been subsequent reports of acute toxicity for these compounds *in vivo* [2], there have not been any reports of eukaryotic genotoxicity. This paper reports an assessment of the genotoxicity of 9 boronic acids, including 8 from the O'Donovan study, using the pan-mechanistic GADD45a genotoxicity reporter assays GreenScreen HC (GFP reporter) and BlueScreen HC (Gaussia luciferase reporter).

The GADD45a assays are used to detect genotoxic hazard and have been described in detail elsewhere. Validation studies have demonstrated a high sensitivity and specificity to all classes of genotoxic carcinogens amongst mechanistically diverse compound collections including mutagens, aneugens and clastogens [3]. Promutagens are also identified from incubation with S9 liver extracts [4,5]. These studies have included compounds from a variety of applicability domains including pharmaceuticals [6], pesticides and herbicides [7]. Assay protocols both with and without S9 metabolic activation have been described, and demonstrated to produce robust reproducible results in inter-laboratory ring trials [8,9]. The GSHC assays are described in the ECVAM INVITTOX protocol number 132 (http://ecvam-dbalm.jrc.ec.europa.eu/). This broad mechanistic coverage and applicability provides a comprehensive assessment of genotoxic hazard in eukaryotic cells, and is therefore an appropriate test for this initial assessment of genotoxic liability within the boronic acids.

Abbreviations: GFP, green fluorescent protein; GADD, growth arrest and DNA damage; GSHC, GreenScreen human cell GADD45a-GFP reporter assay; BSHC, BlueScreen human cell GADD45a-Gaussia luciferase reporter assay.

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2. Materials and methods

2.1. Chemicals

Table 1 lists the compounds tested in this study. They were chosen to complement the O'Donovan study, and selected on the basis of availability.

2.2. GADD45a assays

The GreenScreen HC and BlueScreen HC assays have been fully described elsewhere so are only briefly summarised here. The assay uses human lymphoblastoid TK6 cells. It is a preferred cell line in genetic toxicology because it is p53 wild type, and hence has a properly regulated DNA damage response. Both assays contain plasmids which retain the promoter and the expressed gene sequences, except for a region between translational start and intron 3 which is replaced with a reporter gene: green fluorescent protein in GSHC and *Gaussia* luciferase in BSHC. Control strains for each assay contain an out-of-frame reporter, which allows identification of any compound-related fluorescence or absorbance interference. Compounds were tested in growth medium containing 1% DMSO in a series of 2 fold dilutions across the rows of 96 well microplates (75 μl per well: 9 dilutions for GSHC; 8 dilutions for BSHC). Internal controls included growth media alone (to detect microbial contamination), compound alone (to detect levels of compound colour or fluorescence that might interfere with data collection), and positive controls (methyl methane sulphonate for GSHC, 4-nitroquinoline 1-oxide for BSHC, cyclophosphamide for S9 assays) at two concentrations to confirm proper reporter response. Reporter cells from passage, which had not been allowed to grow beyond one million cells per ml, were collected by centrifugation, re-suspended in double strength assay medium, and 75 μl added to each test sample (reducing top dose to 10 mM, and DMSO concentration to 1%). In the metabolic activation protocols, cells and test samples were incubated for 3 h in the presence of 1% S9 liver extracts from Aroclor treated male Sprague–Dawley rats (Moltox), combined with cofactors. They were then washed, re-suspended in fresh assay medium and incubated for a further 45 h before data collection.

At the end of the assay, reporter outputs (GFP fluorescence, or GLuc luminescence) were recorded together with a measure of culture density: light absorbance (620 nm) for GSHC; thiazole orange (TO) fluorescence for BSHC. TO is a nucleic acid binding dye, detectable using standard fluorescein (FITC) filters (Excitation: 485 nm; Emission: 535 nm). The fluorescence or luminescent reporter output was divided by the measure of cell density to produce a 'brightness' value. In this way the assay discriminates between wells containing low numbers of strongly light-emitting cells and wells containing high numbers of weakly light-emitting cells. A positive result is concluded where exposure within the acceptable toxicity range (≥30% relative suspension growth) produces a significant increase in brightness. This is defined as greater than 3 times the standard deviation derived from studies of toxic and non-toxic non-genotoxins. From the triplicate testing, compounds producing 3/3 or 2/3 positive results were recorded as positive.

3. Results

In the data presented below, all assays fulfilled data acceptance criteria: these include positive controls. Exceptions are noted in the text. Table 1 lists the tested compounds along with CAS numbers, purity, MW and top test concentrations. Fig. 1 shows the dose response graphs for the tested compounds. Table 2 summarises the results.

3.1. GreenScreen HC

In the absence of S9 2,5-Dimethoxyphenylboronic acid (2,5,DMPBA) was the only compound that produced a positive result in all three repeat GSHC tests (LEC 2500 μM), and after 48 h exposure only. 5-Formyl-2-thienylboronic acid produced uninterpretable data due to auto-fluorescence at the wavelength (488 nM) used for GFP detection. This was apparent from fluorescence in the GFP detection channel from both test (GFP expressing) and control (non-GFP expressing) strains.

In the presence of S9, the assay produced negative data for all compounds including the fluorescent 2,5,DMPBA. *m*-Tolylboronic acid produced an isolated positive result in only one of three tests at the maximum tolerated dose (10 mM) and was therefore classified at negative.

3.2. BlueScreen HC results

In the absence of S9, 4 compounds produced positive results: 2,5-dimethoxyphenylboronic acid (LEC 5000 μM), 3,5-difluorophenylboronic acid (LEC 1250 μM), *m*-tolylboronic acid (LEC 2500 μM) and *p*-tolylboronic acid (2500 mM). All produced negative results in the presence of S9.

4. Discussion

This study reports the genotoxicity testing of 9 boronic acid derivatives using the GADD45a GreenScreen and BlueScreen assays, with and without S9 metabolic activation. All produce positive results in the Ames test, and the results presented here suggest that this does not translate into a general eukaryotic liability. When tested using the GreenScreen HC assay, 7 of the 9 produced negative results. 2,5-Dimethoxyphenylboronic acid (2,5-DMPBA) produced the only positive result, and only without S9. 5-Formyl-2-thienylboronic acid is highly coloured and whilst it did not produce interpretable data in the GSHC assay without S9, it was negative in the S9 protocol, where the wash step to remove

Table 1
Compounds tested in this study.

Compound name	CAS no.	Purity(%)	MW	Top test conc. (micromolar)
2-Cyanophenylboronic acid	138642-62-3	≥95.0	146.94	5000
2-Fluoro-6-methoxyphenylboronic acid	78495-63-3	≥95.0	169.95	10000
2,5-Dimethoxyphenylboronic acid	107099-99-0	≥95.0	181.98	5000
3,5-Difluorophenylboronic acid	156545-07-2	≥95.0	157.91	5000
5-Fluoro-2-methoxyphenylboronic acid	179897-94-0	≥95.0	169.95	1250
5-Formyl-2-thienylboronic acid	4347-33-5	≥95.0	155.97	5000
m-Tolylboronic acid	17933-03-8	97	135.96	10000
o-Tolylboronic acid	16419-60-6	≥95.0	135.96	5000
p-Tolylboronic acid	5720-05-8	97	135.96	5000

All compounds were obtained from Sigma–Aldrich.