Carcinogenicity assessment using biosensors: mitomycin-C as a case study

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ABSTRACT

This study enabled an empirical assessment of a model carcinogen and its potency on human receptors. SOS-*lux* biosensors had numerous practical advantages over the traditional assays including procedural simplicity, ease of measurement and *in vivo* analysis without cell disruption. A key advantage of the application of biosensors is their ability to be deployed to genuine samples. This means that while chromatographic analysis requires extraction, purification, clean-up and then analysis, the biosensor can be used in crude and turbid solutions. This could be exclusively for a carcinogenic sensor or for a wider suite of sensors for specific analytes and generic toxicity. Samples could be exposed to a suite of sensors simultaneously allowing a real time response that could prove valuable for the protection of target receptors at a timescale commensurate with intervention. The data presented confirm that the sensors could be fabricated to permit compact analysis and the use of disposable electrodes. Such technologies could transform routine testing and interpretation.

Keywords: sos-*lux* biosensors, *Salmonella* assay, *in vitro* digestion, mutagenicity, bioluminescent bacteria

1 INTRODUCTION

Bacterial biosensors have been constructed to detect a variety of carcinogens [1]. Biosensors for carcinogenicity assessments have been used due to their simplicity and sensitivity [2]. The SOS-*lux* based microbial biosensors have a promoterless *lux*-operon (*luxCDABE*) under control of the SOS-dependant *col* promoter and thus, its synthesis is regulated by the SOS-system [3]. An exposure to a target analyte leads to an increase in the concentration of luciferase and bioluminescence. Subsequently, bioluminescence expression is proportional to the mutagenicity of the agent.

The *Salmonella* mutagenicity assay (Ames assay) is the most widely accepted bacterial assay for the screening and identification of mutagenic compounds [4]. Independent studies have shown a correlation between mutagenicity in the *Salmonella* assay and carcinogenicity in mammals [5]. The assay uses a number of *Salmonella* strains with pre-existing mutations that disable the cells from synthesising histidine, thus inhibiting growth. Fresh mutations at the site of these pre-existing mutations can restore the gene's function and allow the cells to re-synthesis histidine. Enumerations of these mutated colonies in the absence of histidine enables an assessment of carcinogenicity [6]. The assay is reliable but laborious and requires working with *Salmonella* strains that are classified as human pathogen. In this study, Mitomycin-C (MMC) was extracted using a human digestion simulate on procedure (the *in vitro* bioassay), and the carcinogenicity of the extracted samples was assessed using the *Salmonella* assay and SOS-*lux* based microbial biosensors. This enabled an overall appraisal of the optimised assays before working with a complex environment like soil. MMC was then amended into soil samples that extracted using the *in vitro* bioassay followed by comparative carcinogenic assays. Moreover, the effect of the simulated gastrointestinal constituents on the carcinogenicity of MMC was evaluated. The aim of this work was to validate the performance of microbial biosensors for carcinogenicity assessment and consider the relevance of the *in vitro* bioassay in assessing pollutant exposure.

2 MATERIALS AND METHODS

Soil samples of Insch Association/ Insch Series (North-East Scotland) were collected to a depth of 50 cm, passed through a 2 mm stainless steel sieve, and amended with MMC to reach final concentrations of 1, 10 and 50 µg MMC/g dry weight (dw) soil using standard protocols [7]. Soil amended with MMC was extracted by the *in vitro* bioassay and then exposed to the biosensors and the Ames assay. The dose selection of MMC for soil amendment was based upon the dose response curves of MMC which assayed with the Ames assay and the SOS-*lux* biosensors (data not shown). MMC free soil (un-amended soil) was applied as a negative control. MMC was purchased from Sigma (St.Louis, MO, USA), and dissolved in reverse osmosis water.

2.1 The *in vitro* Bioassay

The sequential and the compartmental *in vitro* bioassays were performed for soils amended with MMC. The protocol described by Oomen et al (2003) [8] was used to conduct the sequential *in vitro* bioassay. The compartmental method was achieved by extracting soil samples independently in each digestive compartment (i.e. saliva, gastric and duodenal). Synthetic gastrointestinal juices were prepared as described by Oomen et al. (2003) [8]. In brief, the saliva extraction was accomplished by adding 9 ml of synthetic saliva (pH 6.5 ± 0.2) to 0.6 g soil. The mixture was rotated for 5 min using an end-over-end shaker. The samples were centrifuged for 10 min at 1730 *g* and 37°C ± 2°C. The pellet was discarded and the supernatant removed and stored at 4°C. Gastric extraction was achieved by adding 13.5 ml of the synthetic gastric juice (pH 1.1 ± 0.1) to 0.6 g soil, and the mixture was mixed by end-over-end shaking for 2 h, centrifuged and stored at 4°C. The intestinal extraction was performed by adding 27 ml of the synthetic duodenal juice (pH 7.8 ± 0.2) and 9 ml of the synthetic bile (pH 8.0 ± 0.2) to 0.6 g soil. The mixture was rotated for 2 h and centrifuged as above. The digestive juices were sustained at 37 ± 2°C in a water bath prior to assay.

2.2 Carcinogenicity Assessment Using Biosensors

An aliquot (25 ml) of biosensor strains *E. coli* K12C600 and *E. coli* DPD1718 were grown overnight on LB media at 37°C on an orbital shaker at 150 rpm in the presence of the appropriate antibiotics (50 µg ml^{-1} ampicillin for *E. coli* K12C600 and 30 µg ml^{-1} chloramphenicol for *E. coli* DPD1718) [3,9]. Overnight cultures were diluted 1:50 in LB broth, and grown at 37°C until they reached their pre-optimised CFU (1-2 x 10^9 CFU ml^{-1}). A negative control of 100 µl MilliQ water or 100 µl tested chemical was mixed with 900 µl of overnight culture in 3 ml luminometer cuvettes. Bioluminescence was measured using a Jade bench-top luminometer (Labtech International, Uckfield, UK), over a period of 300 min, with readings taken every 30 min.

2.3 Carcinogenicity Assessment Using the Ames Assay

Salmonella typhimurium TA98, TA100 and TA102 were purchased from Molecular Toxicology Inc. (MD, USA). The strains were maintained and stored according to standard protocols [6]. The Ames assay was accomplished using the standard plate incorporation procedure described by Maron and Ames (1983) [10]. In summary, *Salmonella* strains TA98, TA100 and TA102 were grown overnight in the presence of the appropriate antibiotics (25 µg ml^{-1} ampicillin for TA98 and TA100, and 2 µg ml^{-1} tetracycline for TA102). The cultures were grown until they reached an absorbance of 1.0 at 660 nm (corresponding to 1-2 x 10^9 CFU ml^{-1}). Two ml of melted top agar supplemented with histidine and biotin solution was distributed into sterile glass tubes, and placed in a 45°C water bath. A hundred µl of MMC extracted by the *in vitro* bioassay and 100 µl of the tester strain was added, gently mixed and poured onto the surface of Minimal Glucose Agar plate. The plates were gently tilted and rotated to obtain an even distribution, placed onto a level surface to solidify and incubated at 37°C for 48 h. Following the incubation, the revertant colonies were enumerated on a Gallenkamp colony counter. Appropriate reagent and negative controls [10] were included to enumerate the spontaneous revertants. The assay was carried out using triplicate of each sample and control.

2.4 Data Analysis

Statistical analysis was performed using Minitab 15 for Windows. A result with $p \leq 0.05$ was considered significant. Each sample was tested in triplicate. For the biosensors, a compound was considered a mutagen if there was at least a two fold increase in the bioluminescence response relative to the negative control value [11,12]. If the bioluminescence values decreased during the incubation time, the sample was more likely to be cytotoxic [3]. If bioluminescence was not induced and the cell growth was comparable to that of the untreated control, the test sample was assumed to be neither mutagenic nor cytotoxic. The *two fold increase rule* was applied for the *Salmonella* assay to evaluate the mutagenicity of the tested compounds [10].

3 RESULTS

3.1 Carcinogenicity Assessment of Samples Tested with the Ames Assay in the Aqueous Phase

Carcinogenicity assessment using the Ames assay has shown that *Salmonella* strain TA102 was the most sensitive strain to detect the carcinogenicity of MMC. In comparison, *Salmonella* strains TA98 and TA100 were unresponsive to MMC as the numbers of histidine revertants were not twice the numbers of the spontaneous revertants (data not shown). A significant carcinogenic response was reported for MMC tested with TA102, and extracted in the saliva and the duodenal compartments only (no significant carcinogenic response was reported for MMC extracted in the gastric and in the sequential compartments). To further investigate the reason for that, the pH values of samples extracted in the gastric juice were increased from 1.07 ± 0.10 to 7.4 ± 0.10. Correspondingly, the pH values were increased from 6.8 ± 0.20 to 7.5 ± 0.10 for samples extracted by the sequential *in vitro* bioassay. The data shown no significant carcinogenic response for MMC extracted in the gastric and in the

sequential compartments for the buffered (pH values 7.4 ± 0.10 and 7.5 ± 0.10) and un-buffered (pH values 1.07 ± 0.10 and 6.8 ± 0.20) samples (data not shown).

3.2 Carcinogenicity Assessment of Samples Tested with the Biosensors in the Aqueous Phase

The dose response curve for the biosensor *E. coli* K12C600 exposed to the negative control was significantly different ($p < 0.05$) from samples treated with MMC and extracted in the saliva and the duodenal compartments (data not shown). *E. coli* K12C600 was not significantly induced by samples treated with MMC and extracted by the gastric and the sequential *in vitro* bioassay (data not shown).These results suggested that the biosensor *E. coli* K12C600 was induced by MMC extracted in the saliva and in the duodenal compartments, whereas MMC extracted by the gastric and the sequential *in vitro* bioassays was no longer a carcinogen as it was unable to induce *E. coli* K12C600. The same results were reported for *E. coli* DPD1718 (data not shown) (saliva and in the duodenal compartments but not the gastric and in the sequential compartments). These findings were in-parallel with the results reported with the biosensor *E. coli* K12C600, suggesting that MMC was not a carcinogen once it passed through the gastric compartment of the simulated human gastrointestinal juices.

3.3 Carcinogenicity Assessment of Samples Amended with MMC

The Ames assay responded significantly to Insch soils amended with 10 and 50 µg MMC/g dw soil. No significant carcinogenic response was detected for Insch soil extracted with the gastric and the sequential *in vitro* bioassay (data not shown). These results confirmed that MMC had a carcinogenic effect on *Salmonella* strain TA102 at a concentration of 10 µg MMC /g dw soil and above when extracted in synthetic saliva or duodenal juices. However, MMC was not carcinogenic when extracted in the sequential and the gastric *in vitro* bioassays due to the denaturing of MMC at the extraction pH value.

Insch soil amended with MMC and extracted in the oral compartment had a significant carcinogenic impact on *E. coli* DPD1718 (Fig 1) and *E. coli* K12C600 (Fig 2) at concentrations of 1, 10 and 50 µg MMC/g dw soil. Similarly, significant carcinogenic responses to 10 and 50 µg MMC/g dw soil were observed for soil extracted in the intestinal compartment and assayed with *E. coli* DPD1718 (Fig 1) and *E. coli* K12C600 (Fig 2). The biosensors *E. coli* DPD1718 and *E. coli* K12C600 were unresponsive to samples amended with MMC and extracted by the gastric and the sequential *in vitro* bioassays because MMC denatured at the pH values of these synthetic digestion juices (Fig 1 and 2).

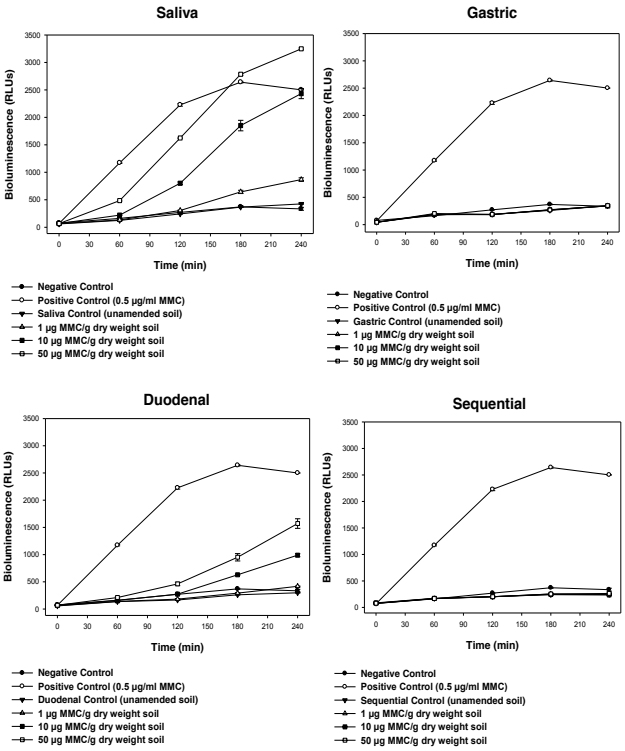

Figure 1: Carcinogenicity response of the biosensor *E. coli* DPD1718 to Insch soils amended with MMC.

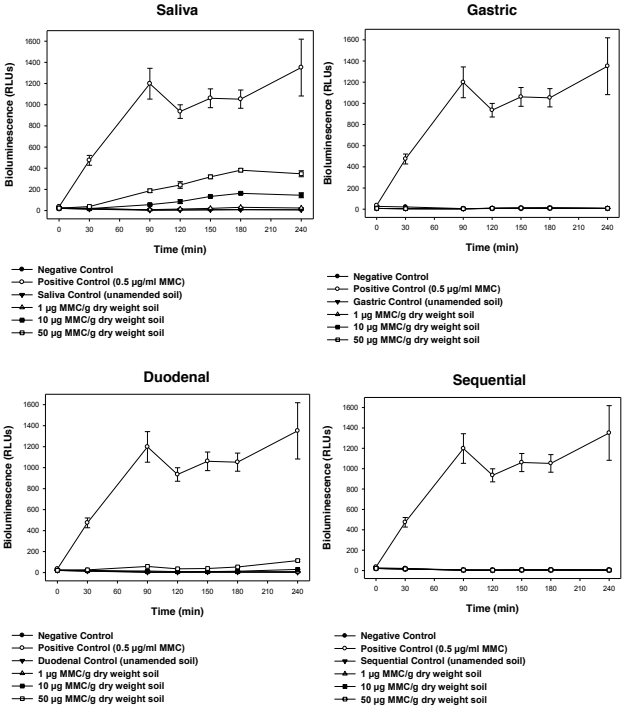

Figure 1: Carcinogenicity response of the biosensor *E. coli* K12C600 to Insch soils amended with MMC.

4 DISCUSSION & CONCLUSION

MMC was selected for this study because it is a highly water soluble compound acknowledged to cause carcinogenicity with both the Ames assay and the biosensor [13]. MMC is a natural occurring compound consisting of a pyrrolo (1,2-a) indole ring system with an aziridine ring. MMC is a potent DNA cross-linker which has a strong ability to crosslink DNA with high efficiency and specificity for the sequence CpG [14]. To interact with DNA, MMC requires enzymatic activation by a one-electron pathway to a semiquinone, or by a two-electron reduction pathway to a hydroquinone [15]. MMC has numerous biological effects in mammalian cells such as: mutagenesis, stimulation of genetic recombination, selective inhibition of DNA synthesis, chromosome breakage and sister chromatid exchange, and induction of the DNA repair system; SOS-response [16].

Salmonella strain TA102 was the most sensitive among the other *Salmonella* strains to detect the carcinogenicity of MMC. Growth inhibition was reported with the *Salmonella* strains TA98 and TA100 when exposed to MMC. The same results were also reported by Maron and Ames [10], who observed inhibition of growth with TA97, TA98 and TA100 when exposed to 2.5 µg/plate of MMC. Consequently, MMC was adopted as a positive control to assess the number of histidine revertants colonies for TA102 [6,10,17]. There is no doubt that the biosensor yielded rapid results when compared with the Ames assay and this is widely acknowledged [18,19]. A limitation is that future developments will require the characterisation and adoption of strong promoters offering genuine relevance with key target receptors [20]. The selection of the particular assays was because they were acknowledged to be responsive to MMC but translation to other chemicals of concern may require a new suite of carcinogenic responsive sensors. It is this point that raised the pertinent issue of sensitivity. The biosensor performance was similar to the Ames assay and this reinforces the value to relate to human protection. The problem is validating the sensitivity against the target receptor and being sure that the response is adequately protective. While to some extent the bioaccessibility *in vitro* procedure verifies the environmental interface there is a need to validate the relative sensitivity of the selected assays and the ability to correlate these with appropriate human assays. Another key advantage of the environmental application of biosensors is their ability to be deployed to genuine samples [20]. This means that while chromatographic analysis requires extraction, purification, clean-up, potentially derivitisation and then analysis, the biosensor can be used in crude and turbid solutions. This could be exclusively for a carcinogenic sensor or for a wider suite of sensors for specific analytes and generic toxicity. While samples could be exposed to a suite of sensors, several aqueous samples can be measured simultaneously allowing a real time response that could prove valuable for the protection of target receptors [18]. The sensors could also be fabricated to permit compact analysis and the use of disposable electrodes. Such technologies could transform routine testing and interpretation.

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