Dose-dependent synergistic and antagonistic mutation responses of binary mixtures of the environmental carcinogen benzo[a]pyrene with food-derived carcinogens.

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Abstract

Cooking food at high temperatures has been shown to produce genotoxic chemicals and there is concern about their impact on human health. Many studies have investigated DNA damage caused by individual chemicals but few have examined the consequences of exposure to mixtures as are found in food. The current study examines the mutagenic response to binary mixtures of benzo[a]pyrene (BaP) with glycidamide (GA), BaP with acrylamide (AC) or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) with GA at concentrations relevant to human exposure (sub-nM). The metabolically competent human MCL-5 cells were exposed to these chemicals individually or in mixtures and mutagenicity was assessed at the thymidine kinase (TK) locus. Mixture exposures gave dose-responses that differed from those for the individual chemicals; for the BaP-containing mixtures, an increased mutation frequency (MF) at low concentration combinations that were not mutagenic individually, and decreased MF at higher concentration combinations, compared to the calculated predicted additive MF of the individual chemicals. In contrast, the mixture of PhIP with GA did not increase MF above background levels. These data suggest that BaP is driving the mutation response and that there is a role for metabolic activation of the chemicals; in mixtures with BaP the increased or decreased MF above or below the expected additive MF is in the order PhIP>AC>GA. Furthermore, competition for nucleophilic guanine at specific gene loci (TK) by activated electrophilic derivatives of PhIP and GA may explain the lack of response above background. Of particular interest when considering the DNA-damaging potential of food is the increase in MF at some low concentration combinations that include BaP. We have previously shown this non-monotonic dose response for mixtures of BaP with PhIP thus these data confirm that this response is not limited only to the BaP/PhIP combination. Moreover, the lack of a mutation response for PhIP with GA relative to the response of the individual chemicals at equivalent doses is interesting and may represent a potential avenue for reducing the risk of exposure to environmental carcinogens; specifically, removal of BaP from the mixture may reduce the mutation effect, although in the context of food this would be significantly challenging.

Introduction

Cooking food at high temperatures has been shown to produce genotoxic chemicals and there is concern about their impact on human health. The consumption of meat, particularly red meat, is positively correlated with human cancer and the cooking of meat produces, amongst others, chemicals such as heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) (Sinha et al. 2005). One of the most abundant HCAs found in cooked food is 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Produced at the parts per billion level (Murray et al. 1993), it is extensively bioavailable to humans consuming cooked meat (ingesting 0.1–15μg PhIP per day) and readily activated by human drug metabolizing enzymes, particularly by cytochrome P450 (CYP) 1A1 and 1A2, to DNA damaging species (Zhao et al. 1994). It is powerfully mutagenic in short-term tests (Knize et al. 2002) and an established rodent carcinogen (Sugimura 1997), shown to induce cancer in the prostate, colon and mammary gland of rats (Ito et al. 1991). Benzo[a]pyrene (BaP) represents one of the best studied and most potent PAH carcinogens and its generation by incomplete combustion of organic substances such as lipids results in the contamination of numerous foodstuffs (Lijinski and Shubik 1964). BaP is metabolised primarily by the CYP1A family to mutagenic derivatives that can form DNA adducts and result in mutation and tumourigenic activity (Cooper and Grover 1990). Through consumption of contaminated food, average human daily exposure to BaP is estimated to be about 1-500ng (IARC 2010). Evidence from numerous experimental studies suggests a positive link between exposure to BaP and cancer in animals and in humans (Sinha et al. 2005). More recently it has been discovered that acrylamide (AC) forms when foods, such as potatoes and cereals, are cooked at temperatures exceeding 100°C, in a reaction between the amino acid asparagine and reducing sugars that are naturally present in these foods (Tornqvist 2005). AC is classified as a probable human carcinogen (IARC 1994) and carcinogenicity has been demonstrated in rat and mouse models (Rice 2005). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has reviewed the toxicity of AC and reported it to be genotoxic and carcinogenic. Margins of exposure (MOE) were considered to be low and may indicate a human health concern (EFSA 2005) with human intake

of AC from the diet estimated to be around 20-50μg per day (Mason and Benford 2007). The genotoxicity of AC is dependent upon its metabolic activation by CYP2E1, with glycidamide thought to be a key activated metabolite (Doerge et al. 2005).

Mixtures of food-derived genotoxic chemicals represent a more realistic exposure scenario, however, published assessment of genotoxic carcinogens, particularly dietary carcinogens, in mixtures is limited. We recently reported that binary mixtures of BaP and PhIP produce dose responses in MCL-5 cells that differ from those of the individual chemicals and from those expected based on current additive predictions (David et al. 2016). Consequently, this follow up study aimed to determine whether such responses were also observed with combinations of BaP with AC or its proposed genotoxic metabolite GA, and to further investigate our hypothesis that BaP is driving these nonmonotonic mutation responses.

Methods

Materials

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich, Poole, UK. RPMI-1640 growth medium (with phenol red, without L-glutamine and histidine), heat-inactivated horse serum (HIHS), L-glutamine, penicillin/streptomycin, and hygromycin B were obtained from Life Technologies, Paisley, UK.

Cell culture

MCL-5 is a human B lymphoblastoid cell line derived from a subpopulation of AHH-1 cells that express a particularly high constitutive level of CYP1A1 activity (Crespi et al. 1991) that has been transfected with two plasmids, one containing two copies of CYP3A4 complementary DNA (cDNA) and one copy of CYP2E1 cDNA, and the other containing one copy each of CYP1A2, CYP2A6 and microsomal epoxide hydrolase cDNA (Crespi et al. 1991).

MCL-5 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) HIHS, 2mM L-glutamine, 100units/ml penicillin, 100µg/ml streptomycin 2mM histidinol and hygromycin B (200µg/ml) called R10. Stock cells were maintained at a concentration of 1-2x10^5/ml and were not cultured for more than the recommended time of 5 weeks (Johnson et al. 2010)

HAT-treatment of cells

To remove background mutants within the MCL-5 population, cells were grown for 3 days in R10 containing HAT (hypoxanthine, aminopterin, thymidine; Hybri-Max™), which is lethal to cells that harbour mutations at the TK locus (Busby et al. 1994). Subsequently, the cells were transferred to media containing HT (hypoxanthine, thymidine; Hybri-Max™) for 24h, then the mutant-depleted cultures were maintained for 4 days in normal media prior to freezing.

TK and HPRT Forward Mutation Assay

Mutation assays used HAT treated cells (50ml at 4 x 10^5 cells/ml) with BaP, PhIP, GA or AC or selected binary mixtures to achieve the final concentrations outlined in Table 1. Dimethylsulfoxide (DMSO) was the negative control and ethyl methanesulfonate (EMS; 10µg/ml) the positive control for all experiments. Mutation data were considered acceptable provided that the relative total growth (RTG) and mutant frequency (MF) for both DMSO and EMS controls complied with historical data and that RTG additionally complied with Organisation for Economic Co-operation and Development (OECD) guidelines (data not shown) (OECD 1997). Published methodology (Clements 2000) was followed, with some optimisations. Cells were treated for 24h in RPMI-1640 containing all supplements but reduced serum (5% (v/v) HIHS), at 37°C, 5% CO_2. Following treatment, cells were adjusted to 4x10^5 cells/ml and subcultured daily for 2 further days to determine the relative suspension growth (RSG) (Clements 2000). On the third day cells were plated at 10 cells/well in 2 x 96-well plates to determine cloning efficiency (CE) and 20 000 cells/well in 3 x 96 well plates in trifluorothymidine (TFT; 4µg/ml) to determine the thymidine

kinase (TK) MF. Plates were incubated for 21 days at 37°C, 5% CO_2 prior to colony scoring to determine the MF. A minimum of four replicate cultures were used per treatment. RTG was calculated to estimate cytotoxicity and MF is expressed as mutants/10^6 viable cells (Clements 2000).

Ethoxyresorufin-O-deethylase **(EROD)**

Ethoxyresorufin-*O*-deethylase (EROD; an indicator of CYP1A activity) was measured as the conversion of 7-ethoxyresorufin (7-ER) to resorufin. Cell suspensions (10ml at 4 x 10^5 cells/ml) were treated with a mixture of BaP with AC for 24h following which 3x10^6 cells were collected for EROD activity analysis by centrifugation (200x*g*, 5 minutes, RT), washed once in phenol red-free serum-free RPMI-1640 media (R0) and re-suspended in 1ml R0 media in 24 well plates, 8μM 7-ER added, and the plate incubated for 90 minutes at 37°C. Fluorescence was measured at λexcitation 560nm and λemission 590nm every 10 minutes using a fluorescence plate reader (POLARstar Galaxy, BMG Lab Technologies). Activity was expressed as pmol resorufin produced/min/10^6 cells using a resorufin standard curve.

Protein Determination

Cells (3x10^6) collected by centrifugation (200x*g*, 5 minutes, RT) were treated with RIPA buffer with the addition of Halt protease inhibitor cocktail (Invitrogen) for 30 minutes on ice. The lysate was clarified by centrifugation (8000 x *g,* 10 min, 4°C), the supernatant collected and stored at -20°C. Protein concentration of the lysate was determined using the bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific) following manufacturer's instructions.

Statistical analysis of mutation data to determine synergy/antagonism

Median Effect Plot and Combination Index (CI)

Data were analysed by the method of Chou (2006) as previously described by David et al. (David et al. 2016) using background-corrected MF values for all calculations. Median effect plots of log(dose) vs log(fa/fu), where fa is the fraction affected (MF/1e6 viable cells) and fu is 1e6-fa, were drawn for each individual chemical to obtain the slope (m), the median effect dose (Dm, calculated as the antilog of the x intercept when y=0) and the Pearson correlation coefficient (r), which signify the shape of the dose-effect curve, the potency (IC_{50}), and the conformity of the data to the mass action law, respectively. From these the doses of the individual chemicals alone required to produce the mixture effect were calculated using equation 1:

$$Dx = Dm[\frac{fa(mix)}{1-fa(mix)}]^{1/m} \quad (1)$$

The combination index was calculated using equation 2:

$$CI = \frac{D_1}{Dx_1} + \frac{D_2}{Dx_2} \quad (2)$$

where D_1 and D_2 are the concentrations of the individual chemicals used in the mixture and subscripts 1 and 2 refer to the two components of the mixture.

For the CI calculation, the value D was also calculated using equation 1. While D represents the dose of the individual chemical used in the mixture, Chou (2006) states that *'the dose and the effect are interchangeable since the dose (D) for any given effect (fa) can be determined if the values for Dm and m are known'.* Since Dm and m are obtained from the median effect plot, from which Dx values are also derived, it was noted that calculating D based on these values gave modified doses, thus we have adjusted D to reflect the median effect plot.

Synergism and antagonism are determined from the CI and are subdivided into nearly additive (0.9-1.1), slight synergism/antagonism (0.85-0.9/1.1-1.2), moderate synergism/antagonism (0.7-0.85/1.2-1.45), synergism/antagonism (0.3-0.7/1.45-3.3), strong synergism/antagonism (0.1-0.3/3.3-10) and very strong synergism/antagonism (<0.1/>10) (Chou 2006).

Interaction factor (IF)

Data were also analysed using the interaction factor (IF), which was calculated following Danesi et al. (2012) and as previously described by David et al. (David et al. 2016) using background-corrected MF values for all calculations. The IFs were calculated using equation 3:

$$IF = G_1G_2 - G_1 - G_2 + C \quad (3)$$

where G_1G_2 is the MF obtained in the treatment with the combination, G_1 and G_2 are the MF obtained in the treatment with the individual chemicals alone, and C is the MF obtained in the control. A negative IF denotes antagonism, a positive IF denotes synergism and a zero IF denotes additivity.

The standard error of the mean (SEM) of IF was calculated as described by Danesi et al., (2012) using equation 4:

$$SEM = \sqrt{(SEM_{\mathrm{G1G2}})^2 + (SEM_{G1})^2 + (SEM_{G2})^2 + C} \quad (4)$$

where SEM_{G1G2} is the SEM for the mixture.

Independent action (IA)

Concentration-response relationships of mixtures of compounds are predicted based on concentration-response data for the individual mixture components, assuming additivity (Rajapakse et al. 2001). Synergism and antagonism can be defined as deviations from the expected effects, with synergistic mixtures showing higher, and antagonistic mixtures lower, responses than predicted. When predictions are met the combined response is additive (Berenbaum 1989). Independent action (IA) represents the situation where compounds act on different subsystems within an organism, possibly involving different sites and modes of action (Rajapakse et al. 2001). Since the chemicals used in this study have different modes of action, we have used this method (previously described by David et al. (David et al. 2016)) for determining the expected response.

IA can be calculated using equation 5 as described by Berenbaum (1989):

$$E(da, db) = E(da) + E(db) - E(da)E(db) \quad (5)$$

where E(da, db) is the fractional effect of the mixture, and E(da) and E(db) is the fractional effect of the individual chemicals. In this equation, the fractional effect E is used as a substitute for the probability of occurrence of an event, and fractional lack of effect (Berenbaum 1989). When applying this model to our assay, a maximal effect has to be defined (Rajapakse et al. 2001). In the current study, the fractional effect E is the MF, which is expressed as number of mutants per 1e6 viable cells, thus we assume the unit of assessment is the cell and thus the maximal effect is 1e6 mutants per 1e6 cells.

IA was calculated using equation 6, based on that employed by Abendroth et al. (2011):

$$IA = E_1 + E_2 - \left(\frac{E_1 E_2}{10^6}\right) \quad (6)$$

where E_{1+2} is the IA predicted mixture percent response, E_1 is the percent response for chemical 1 and E_2 is the percent response for chemical 2.

Results

TK forward mutation assay for individual chemicals

The TK forward mutation assay were used to investigate the mutagenicity of BaP, PhIP, AC and GA in MCL-5 cells. For all individual chemicals, the concentrations that were used were chosen to cover a range of values from typical human dietary exposure (< 10^{-8} M) (IARC 2010; Sinha et al. 2005) to high concentrations that induce a high mutant frequency (Felton et al. 2002; Yadollahi-Farsani et al. 1996). A non-linear dose-response was observed for all chemicals. Exposure to BaP and PhIP (previously reported) produced statistically significant increases in MF at TK at doses ≥2.5x10^{-7} and ≥5x10^{-5} M, respectively (Table 2; Figure 1) (David et al. 2016).

AC significantly increased MF at 4x10^{-3}M (Figure 1A) while treatment with GA, the proposed genotoxic metabolite of AC, produced a statistically significant increase in MF at 10^{-4}M at TK (Figure 1C). The relative mutagenic potency of all four chemicals examined was BaP>PhIP>GA>AC, consistent with reported literature (IARC 2010; Yadollahi-Farsani et al. 1996).

Mutation frequency at TK loci for binary mixtures

BaP with GA

Based on the relative concentrations of BaP (the more powerful mutagen) and GA, the TA locus mutation dose-response for the mixtures did not follow simple additivity and was not linear (Figure 2A; Table 2).

In general, MF was increased at low concentration mixtures and was not enhanced for high

concentration mixtures relative to the expected response if the MF for the individual chemicals was additive. For example, a statistically significant increase in MF was observed for the combinations 10^{-7}M BaP with 10^{-5}M GA (TK MF = 42.8 ± 6.7) whereas these concentrations of BaP and GA alone did not significantly increase the MF (TK MF = 1.3 ± 1.2 and 0.8 ± 0.1 respectively) (Figure 2 and Table 2). In contrast, for the combination 10^{-5}M BaP with 10^{-4}M GA (TK MF = 48.0 ± 6.6) the MFs were lower than anticipated given that these concentrations of BaP and GA alone produced significant increases in MF (TK MF = 76.9 ± 10.5 and 11.8 ± 1.0) (Figure 2 and Table 2). The combination of 10^{-5}M BaP with 10^{-5}M GA significantly reduced the RTG compared to that of the individual chemicals (Table 2).

BaP with AC

AC alone did not produce statistically significant increases in MF at concentrations below $4x10^{-3}$M. Yet a statistically significant increase in MF was observed for all AC plus BaP combinations tested. For example, a TK MF of 23.8 ± 2.2 was observed for 10^{-7}M BaP with $4x10^{-6}$M AC, which is higher than would be expected if the MF for the individual chemicals was additive, whereas these concentrations of BaP and AC alone did not significantly increase the MF (TK MF = 1.3 ± 1.2 and 0.8 ± 0.1 respectively) (Figure 3 and Table 2). In contrast, for 10^{-5}M BaP with $4x10^{-6}$M AC the response was lower than would be expected if the MF for the individual chemicals was additive (TK MF = 19.5 ± 2.3), whereas this concentration of BaP alone produced a significant increase in MF (TK MF = 76.9 ± 10.5) (Figure 3 and Table 2).

PhIP with GA

To test the hypothesis that BaP is driving the mutation response in the binary mixtures, cells were exposed to selected concentration combinations of PhIP with GA. At TK no increase above the predicted additive MF was observed for any of the combinations tested with the exception of $7.5x10^{-5}$M GA with 10^{-4}M PhIP (TK MF = 62 ± 10.7) (Figure 4A; Table 2).

EROD

AC has been reported to induce CYP1A activity, so we measured EROD activity following exposure to AC, with or without BaP to determine whether AC could induce CYP1A activity or affect BaP-induction of EROD activity. The results showed that AC did not induce EROD activity in MCL-5 cells, nor did it affect BaP induction of EROD activity (Figure 5).

Statistical analysis of the binary mixture data

Three methods of statistical determination of interaction were employed to assess whether the combinations tested were additive, synergistic or antagonistic as previously described(David et al. 2016); The Median Effect Equation derived from the mass action law principle (Chou 2006), the interaction factor (IF) (Schlesinger et al. 1992), (Danesi et al. 2012), and response addition based on independent action (IA), (Rajapakse et al. 2001).

For BaP with GA, the results from all three methods at the TK locus show synergistic interactions at doses of BaP $\geq 10^{-7}$M with doses of GA $\geq 10^{-5}$M and antagonism for BaP 10^{-5}M with doses of GA $\geq 10^{-5}$M (Table 3).

For BaP with AC, at TK a synergistic interaction was identified for 10^{-7}M BaP with $4x10^{-6}$M AC and antagonism for 10^{-6}M or 10^{-5}M BaP with $4x10^{-6}$M AC.

For PhIP with GA, all three methods indicated interactions that were not different from additive or antagonistic for the majority of combinations of PhIP with GA (Table 3), although synergistic effects were noted at high concentration GA mixtures (>7.5 x 10^{-5} M GA).

Discussion

Cooking food at high temperatures has been shown to produce DNA damaging chemicals. Many studies have investigated DNA damage caused by individual chemicals but few have examined the consequences of exposure to mixtures as are found in food. We have previously shown that

mixtures of BaP with PhIP produce non-monotonic mutation profiles(David et al. 2016), thus this follow up study aimed to further investigate this phenomenon by combining these mutagens in binary mixtures with other direct and indirect acting genotoxins. It is also worth noting that our previously reported data with BaP and PhIP mixtures are consistent with the report by Jamin et al.,(Jamin et al. 2013) who showed combined genotoxicity and an increased level of PhIP-DNA adducts at a single mixture combination of BaP 10^{-6} M + PhIP 10^{-5} M.

Mutagenicity of individual chemicals

As we have previously shown, the TK forward mutation assays with BaP indicate that this compound induces a statistically significant increase in MF at concentrations ≥10^{-7}M. Additionally, PhIP significantly increased MF at concentrations ≥10^{-5}M at the TK locus (David et al. 2016). In the current study, AC only significantly increased MF at 4x10^{-3}M at TK, while GA induced a statistically significant increase in MF at 10^{-4}M at TK. In line with our findings, it has previously been reported that AC induces mutations at the TK but not HPRT locus, suggesting AC primarily induces large-scale chromosomal changes (Koyama et al. 2006). AC is known to be metabolised by CYP2E1 to GA, which induces point mutations that should be detectable in the TK assay. Our results suggest that minimal or no metabolic activation of AC is occurring in MCL-5 cells, despite these cells expressing CYP2E1. In support of our data it has been reported that AHH-1 cells (parent cell line of MCL-5) transfected with a plasmid containing CYP2E1 (h2E1v2 cells) show a genotoxic response to N-di-N-butylnitrosamine (DMN), a representative CYP2E1 substrate, but not AC (Koyama et al. 2011).

Of interest is that the combination of 10^{-7}M BaP with 10^{-5}M GA gave a significant increase in MF, which was not observed for these respective concentrations of the individual chemicals alone. This interaction was synergistic as analysed by the median effect, IF and IA approaches (Table 3, Figure 2). In contrast, 10^{-5}M BaP with 10^{-4}M GA gave a lower MF than would be expected for an additive response despite these concentrations producing a significant increase in MF individually. BaP requires metabolic activation by CYP1A1 whereas GA is the activated metabolite

of AC and thus does not require metabolism to exert its genotoxic effect. As such, the interaction between these chemicals resulting in the synergistic induction of MF is unclear.

AC only gave a positive response in the mutation assay at $4x10^{-3}$M. The lower dose of $4x10^{-6}$M in combination with 10^{-7}M BaP produced a synergistic induction of MF as analysed by the median effect, IF and IA approaches, while 10^{-5}M BaP with $4x10^{-6}$M AC gave a lower than predicted additive MF. While AC does require metabolic activation, this occurs via CYP2E1, and as such is not expected to be influenced by BaP. A recent study by Sen et al., (2012) showed AC induced EROD and MROD activities in HepG2 cells, however AC did not affect EROD in MCL-5 cells in the current study, nor was there any effect on BaP-induced EROD activity. However, it should be noted that this was following a 24h treatment in our study, whereas Sen et al. (2012) used a 48h treatment. These results suggest that an alternative mechanism to one involving modulation of CYP2E1 activity is involved in the synergistic interaction, but more investigation is required to elucidate this.

Regarding the lower than predicted MF observed for the high concentration combinations, we have shown that for mixtures of BaP with PhIP, cell cycle arrest driven by PhIP, apparently independent of BaP, is responsible(David et al. 2016). Based on these data, the antagonistic response observed for 10^{-5}M BaP with GA and AC suggests some involvement of DNA repair, but that possibly these pathways are activated to a lesser extent than for BaP in combination with PhIP. This requires further investigation.

In contrast to our results for combinations with BaP, no induction of MF above the expected additive response was observed at any of the combinations of PhIP with GA tested at TK. This supports the conclusion that BaP is driving the mutation response in the mixtures we have tested.

Conclusion

In conclusion, the mutation responses for the binary mixtures tested differ considerably from those predicted by the IA model of additivity. An important finding from the current study is that combining non-measurably mutagenic concentrations of BaP with non-mutagenic

concentrations of other genotoxicants, either direct acting or those requiring metabolic activation, produces a significant increase in MF at TK. The decrease in MF at high concentration combinations at TK is of interest as it implies that in combination at these concentrations, these chemicals are less mutagenic. In contrast to the results for binary mixtures containing BaP, no increase in MF above background was observed for mixtures of PhIP with GA at TK. This suggests that BaP is driving the mutation response in those mixtures where a non-monotonic dose response is observed. The increase in MF at low concentration combinations of mixtures involving BaP is of significance when considering the genotoxic potential of food since these concentrations are more relevant to human exposure.

We have previously shown that binary mixtures of BaP with PhIP produce a similar non-monotonic dose response(David et al. 2016) as observed for the combinations with BaP tested here, thus these data are important in confirming that this phenomenon is not confined only to the combination of BaP with PhIP. Moreover, these data highlight the need for further investigation into the mutation profile of other binary mixtures, both containing BaP and extended to include other PAHs and environmental genotoxic carcinogens. In contrast, the binary mixture of PhIP with GA did not increase MF above background levels. This warrants further investigation, as our data suggest that for mixtures of genotoxic chemicals that act synergistically (those that include BaP), removing one component (BaP) will disproportionately reduce the effect, thus this may lead to potential avenues for reducing the risk of exposure to environmental carcinogens. We recognise, however, that achieving this at levels of the compound that are relevant to human exposure and are naturally found in food will be significantly challenging.

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Figure Legends

Figure 1. Effect of BaP, PhIP, acrylamide (AC) and glycidamide (GA) on mutation frequency (MF) at the TK locus.

Background corrected MF at TK following a 24h treatment of MCL-5 cells with AC (A) or GA (B). Historical controls: TK: DMSO (negative control) 13.3±1.9 and EMS (positive control): 99.4±9.5; MF/1e6 viable cells ± 95% confidence interval (n=>64). Data shown are means ± SEM, n=3-12. Significance compared to DMSO control (one-way ANOVA with Dunnett's post-test; * P≤0.05, ** P≤0.01).

Figure 2. Effect of binary mixtures of BaP with glycidamide (GA) on mutation frequency (MF) at the TK locus.

Background corrected MF at TK locus following 24h treatment of MCL-5 cells with binary mixtures of BaP with GA. Open bars are predicted MF based on additivity and closed bars are actual MF. Historical controls: TK: DMSO (negative control) 13.3±1.9 and EMS (positive control): 99.4±9.5 MF/1e6 viable cells ± 95% confidence interval (n=>64). Data shown are means, n=3-12.

Figure 3. Effect of binary mixtures of BaP with acrylamide (AC) on mutation frequency (MF) at the TK locus.

Background corrected MF at TK locus following 24h treatment of MCL-5 cells with binary mixtures of BaP with AC. Open bars are predicted MF based on additivity and closed bars are actual MF. Historical controls: TK: DMSO (negative control) 13.3±1.9 and EMS (positive control): 99.4±9.5 MF/1e6 viable cells ± 95% confidence interval (n=>64). Data shown are means, n=3-12.

Figure 4. Effect of binary mixtures of PhIP with glycidamide (GA) on mutation frequency (MF) at the TK locus.

Background corrected MF at TK locus following 24h treatment of MCL-5 cells with binary mixtures of PhIP with GA. Open bars are predicted MF based on additivity and closed bars are actual MF. Historical controls: TK: DMSO (negative control) 13.3±1.9 and EMS (positive control): 99.4±9.5 MF/1e6 viable cells ± 95% confidence interval (n=>64). Data shown are means, n=3-12.

Figure 5. Effect of acrylamide (AC) alone or in combination with benzoapyrene (BaP) on CYP1A activity.

Ethoxyresorufin-O-deethylase (EROD) activity following 24h treatment of MCL-5 cells with AC alone or in combination with BaP. Data are means ± SEM, n=3. Significance compared to the DMSO control (one-way ANOVA with Dunnett's post-test; * P≤0.05).

Tables

Table 1 Concentrations of genotoxicants used for the mutation assays

Treatment	Experiment	Final concentration (M)[1]
BaP	First dose response	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}
	Focused dose response	$2.5x10^{-7}$, $7.5x10^{-7}$, $2.5x10^{-6}$, $7.5x10^{-6}$
PhIP	First dose response	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, $5x10^{-5}$
	Focused dose response	$5x10^{-6}$, 10^{-5}, $2.5x10^{-5}$, $5x10^{-5}$, $7.5x10^{-5}$, 10^{-4}
AC	First dose response	10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}
	Extended dose response	10^{-3}, $2x10^{-3}$, $3x10^{-3}$, $4x10^{-3}$, ($5x10^{-3}$, $6x10^{-3}$)*
GA	First dose response	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}
	Focused dose response	10^{-5}, $1.5x10^{-5}$, $2.5x10^{-5}$, $5x10^{-5}$, $7.5x10^{-5}$, 10^{-4}
BaP+GA	Initial combination set	10^{-9}+10^{-9}, 10^{-7}+10^{-5}, 10^{-7}+10^{-4}, 10^{-6}+10^{-6}, 10^{-5}+10^{-5}, 10^{-5}+10^{-4}
PhIP+GA	Initial combination set	10^{-9}+10^{-9}, 10^{-6}+10^{-6}, $5x10^{-5}$+10^{-5}, $5x10^{-5}$+10^{-4}, 10^{-4}+10^{-5}, 10^{-4}+10^{-4}
	Follow up combination set	$7.5x10^{-5}$+$7.5x10^{-5}$, $7.5x10^{-5}$+10^{-5}, $5x10^{-5}$+$7.5x10^{-5}$, ($7.5x10^{-5}$+10^{-4})*, 10^{-4}+$7.5x10^{-5}$
BaP+AC	Initial combination set	10^{-7}+$4x10^{-6}$, (10^{-7}+$4x10^{-3}$)*, 10^{-6}++$4x10^{-6}$, 10^{-5}+$4x10^{-6}$, (10^{-5}+$4x10^{-3}$)*

[1]For mixtures, the concentrations are given in the order shown under treatment.
* Toxic concentrations, therefore these were not carried through to the mutation assay.

Table 2 Relative Total Growth (RTG) and background-corrected mutation frequency (MF/1e6 viable cells) at the thymidine kinase (TK) locus following treatment with different concentrations of benzo[a]pyrene (BaP), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), glycidamide (GA) or acrylamide (AC) alone, or selected combinations of GA or AC with BaP or PhIP.

Treatment		RTG[a]	TK MF[b]
BaP[c]	10^{-10}M	98.7 ± 0.7	0.1 ± 1.3
	10^{-9}M	95.1 ± 3.8	0.1 ± 1.5
	10^{-8}M	87.6 ± 5.9	0.1 ± 1.8
	10^{-7}M	85.6 ± 6.1	1.3 ± 1.2
	2.5x10^{-7}M	85.6 ± 7.9	30.1 ± 4.3**
	7.5x10^{-7}M	74.1 ± 1.4	44.5 ± 4.7***
	10^{-6}M	74.2 ± 4.0	46.5 ± 6.9***
	2.5x10^{-6}M	85.9 ± 6.5	54.4 ± 3.6***
	2.5x10^{-6}M	64.4 ± 0.4	61.4 ± 8.5***
	10^{-5}M	56.4 ± 7.7	76.9 ± 10.5***
PhIP[c]	10^{-9}M	98.0 ± 3.9	0.7 ± 0.4
	10^{-8}M	113.2 ± 3.2	0.1 ± 1.3
	10^{-7}M	112.6 ± 6.8	5.6 ± 2.4
	10^{-6}M	114.2 ± 5.9	2.1 ± 1.6
	10^{-5}M	103.9 ± 2.9	5.1 ± 1.5
	5x10^{-5}M	89.2 ± 4.9	7.0 ± 1.6**
	7.5x10^{-5}M	78.6 ± 4.7	8.8 ± 1.2*
	10^{-4}M	83.0 ± 5.4	7.9 ± 2.4**
GA	10^{-9}M	102.04± 9.5	0.6± 1.1
	10^{-8}M	89.5± 9.8	0.1± 1.8
	10^{-7}M	96.2± 11.5	1.7± 2.1
	10^{-6}M	98.5± 10.9	0.6± 1.0
	10^{-5}M	95.6± 8.3	0.8± 0.1
	1.5x10^{-5}M	76.5± 6.5	4.1± 3.5
	2.5x10^{-5}M	68.4± 3.7	0.5± 2.9
	5x10^{-5}M	60.6± 5.4	1.9± 3.5
	7.5x10^{-5}M	62.3± 2.6	5.5± 3.3
	10^{-4}M	70.7±3.3	11.8*±1.0
AC	10^{-7}M	91.6± 7.3	0.8± 2.1
	10^{-6}M	92.9± 5.2	2.2± 1.7
	4x10^{-6}M	97.9±7.6	0.1±2.0
	10^{-5}M	81.4± 1.0	0.5± 1.6
	10^{-4}M	92.8± 6.8	1.5± 1.4
	10^{-3}M	79.8± 6.2	0.2± 0.7
	2x10^{-3}M	93.9± 10.3	1.1± 2.8
	3x10^{-3}M	56.4± 3.1	0.1± 2.2
	4x10^{-3}M	20.8± 3.1	17.5*± 5.3
BaP + GA	10^{-9}M+10^{-9}M	93.7± 0.6	0.1± 3.2
	10^{-7}M+10^{-5}M	76.3± 7.5	42.8**± 6.7
	10^{-7}M+10^{-4}M	82.5± 5.9	53.1***± 10.0
	10^{-6}M+10^{-6}M	72.4± 4.9	53.7***± 4.1
	10^{-5}M+10^{-5}M	38.8± 1.9[*]	62.6***± 9.2
	10^{-5}M+10^{-4}M	55.6± 5	48.0***± 6.6
BaP + AC	10^{-7}M+4x10^{-6}M	90.7±5.2	23.8***±2.2
	10^{-6}M+4x10^{-6}M	66.2±3.3[**]	23.7***±3.6
	10^{-5}M+4x10^{-6}M	43.1±2.9[***]	19.5***±2.3
PhIP + GA	10^{-9}M+10^{-9}M	126.1±8.5	0.1±1.5
	10^{-6}M+10^{-6}M	119.6±8.5	0.1±1.2
	5x10^{-5}M+10^{-5}M	111.5±4.8	0.1±1.0
	5x10^{-5}M+7.5x10^{-5}M	66.0±10.5	24.3±10.4
	5x10^{-5}M+10^{-4}M	82.0±3.4	7.1±2.6
	7.5x10^{-5}M+10^{-5}M	86.2±3.4	0.1±8.8
	7.5x10^{-5}M+7.5x10^{-5}M	87.6±5.9	15.8±3.5
	7.5x10^{-5}M+10^{-4}M	62.4±5.5	22.0±5.9
	10^{-4}M+10^{-5}M	93.4±6.9	1.8±3.3
	10^{-4}M+7.5x10^{-5}M	45.6±3.1[***]	62.8***±10.7
	10^{-4}M+10^{-4}M	80.3±4.2	11.0*±3.1

[a]RTG values are % means ± Standard Error of the Mean (SEM), n=3-8

[b]Data are presented as means ± Standard Error of the Mean (SEM), n=3-8; Historical controls: TK: DMSO: 13.3±1.9 and EMS (positive control): 99.4±9.5; HPRT: DMSO: 5.7±2.1 and EMS (positive control) 53.1±21.8 MF/1e6 viable cells ± 95% confidence interval; Significance compared to the DMSO control calculated using one-way ANOVA with Dunnett's post-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

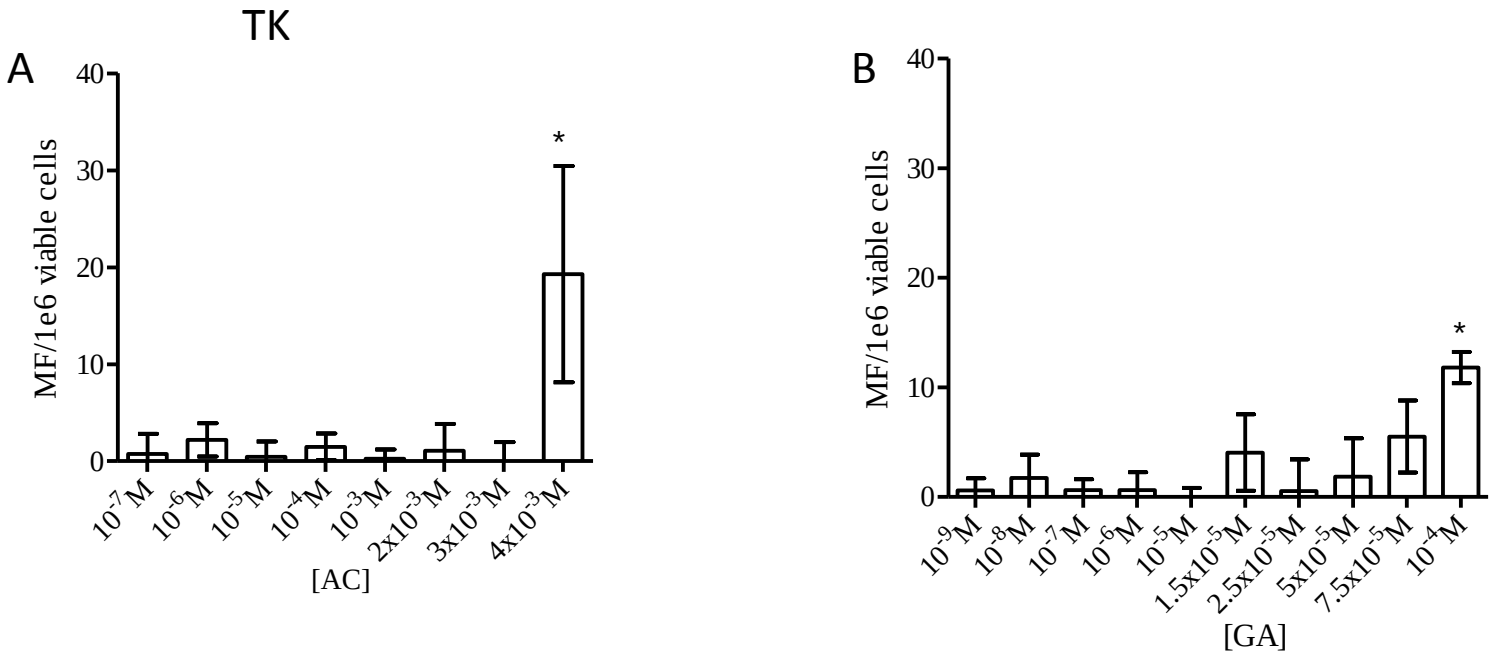
[c]BaP and PhIP RTG and TK MF data as published in David et al. (2016)

RTG of mixture compared to the chemical alone: Significance calculated using one-way ANOVA with Dunnett's post-test ([*] $P \leq 0.05$, [**] $P \leq 0.01$, [***] $P \leq 0.001$).

Table 3 Analysis of the mutation frequency data at the TK and HPRT loci for BaP and GA in binary mixture by the Median Effect equation and the Combination Index Theorum (CI), Interaction Factor (IF) or Independent Action (IA).

Mixture	fa[b]	CI[c]	Mechanism (CI)[d]	IF ± SEM[e]	Mechanism (IF)[e]	Predicted MF with IA[f]	Mechanism (IA)[g]
TK locus							
BaP+GA							
10^{-9}+10^{-9}	0.10 ± 3.25	468.03	SA	-0.6 ± 4.32	NDAd	0.7	NDAd
10^{-7}+10^{-5}	42.77 ± 6.69	0.01	SS	41.38 ± 4.85	S	1.39	S
10^{-7}+10^{-4}	53.15 ± 10.00	0.01	SS	40.05 ± 6.95	S	13.10	S
10^{-6}+10^{-6}	53.66 ± 4.09	0.82	MS	6.58 ± 8.37	S	47.08	S
10^{-5}+10^{-5}	62.63 ± 9.24	1.34	MA	-14.00 ± 12.09	A	77.06	A
10^{-5}+10^{-4}	48.01 ± 6.61	1.94	MA	-40.76 ± 9.09	A	88.77	A
BaP+AC							
10^{-7}+4x10^{-6}	23.81±2.2	0.02	SS	22.42±4.03	S	1.39	S
10^{-6}+4x10^{-6}	23.72±3.6	2.58	A	-22.84±8.43	A	46.56	A
10^{-5}+4x10^{-6}	19.5±2.3	6.90	SA	-57.55±5.80	A	77.06	A
PhIP+GA							
10^{-9}+10^{-9}	0.10 ± 1.52	5.00E+04	SA	-1.23 ± 3.64	A	1.33	A
10^{-6}+10^{-6}	0.10 ± 1.21	1.08E+05	SA	-2.58 ± 4.46	A	2.68	A
5x10^{-5}+10^{-5}	0.10 ± 1.05	2.13E+06	SA	-5.63 ± 5.18	A	6.15	A
5x10^{-5}+7.5x10^{-5}	24.26 ± 11.08	0.0074	SS	11.73 ± 12.58	S	11.56	S
5x10^{-5}+10^{-4}	7.06 ± 2.56	23.798	SA	-10.80 ± 5.83	A	17.87	A
7.5x10^{-5}+10^{-5}	3.95 ± 6.86	16.88	SA	-4.48 ± 7.69	A	8.86	A
7.5x10^{-5}+7.5x10^{-5}	15.76 ± 3.38	0.13	SS	-0.21 ± 4.84	NDAd	14.27	NDAd
7.5x10^{-5}+10^{-4}	22.05 ±6.41	0.06	SS	1.83 ± 7.35	S	20.57	S
10^{-4}+10^{-5}	1.81 ± 3.34	1101.65	SA	-10.87 ± 5.11	A	13.11	A
10^{-4}+7.5x10^{-5}	62.76 ± 9.06	0.0037	SS	42.54 ± 10.33	S	18.52	S
10^{-4}+10^{-4}	11.03 ± 3.15	3.32	SA	-13.80 ± 5.09	A	24.82	A

[a] Molar concentration (BaP is shown first); [b]Fraction affected (fa) is background corrected mutation frequency/1e6 viable cells±SEM; [c]Combination Index (CI)=$(D_1/Dx_1)+(D_2/Dx^2)$; D_1, D_2 are the concentrations used in the mixture and Dx_1, Dx_2 are the concentrations of chemical alone to achieve the mixture effect; [d]Synergism and antagonism are subdivided into nearly additive (NAd, 0.9-1.1), moderate synergism/antagonism (MS, 0.7-0.90/MA,1.1-1.45), synergism/antagonism (S,0.3-0.7/ A, 1.45-3.3), strong synergism/antagonism (SS, <0.1-0.3/ SA, 3.3->10), (17); [e]Interaction factor (IF)=G_1G_2-G_1-G_2+C and SEM. A negative IF=antagonism (A), positive IF=synergism (S), 0=not different from additive (NDAd); [f]Independent Action (IA)=MF_1+MF_2-[$(MF_1MF_2)/10^6$]; MF_1 and MF_2 = individual MF, MF_1MF_2 = product of individual MFs.; [g]Mechanism deduced by comparison of predicted MF to the actual MF (fraction affected); Synergism (S), Antagonism (A), Not different from additive (NDAd). Observed and predicted MF response compared using a t test with Bonferroni multiple comparisons test (* P≤0.05, *** P≤0.001). Variance surrounding the expected MF was assumed to equal the variance for the observed data (Abendroth et al., 2011) (fa column).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30
20
10
0
*
10-7M
10-6M
10-5M
10-4M
10-3M
2x10-3M
3x10-3M
4x10-3M
[AC]
B
MF/1e6 viable cells
40
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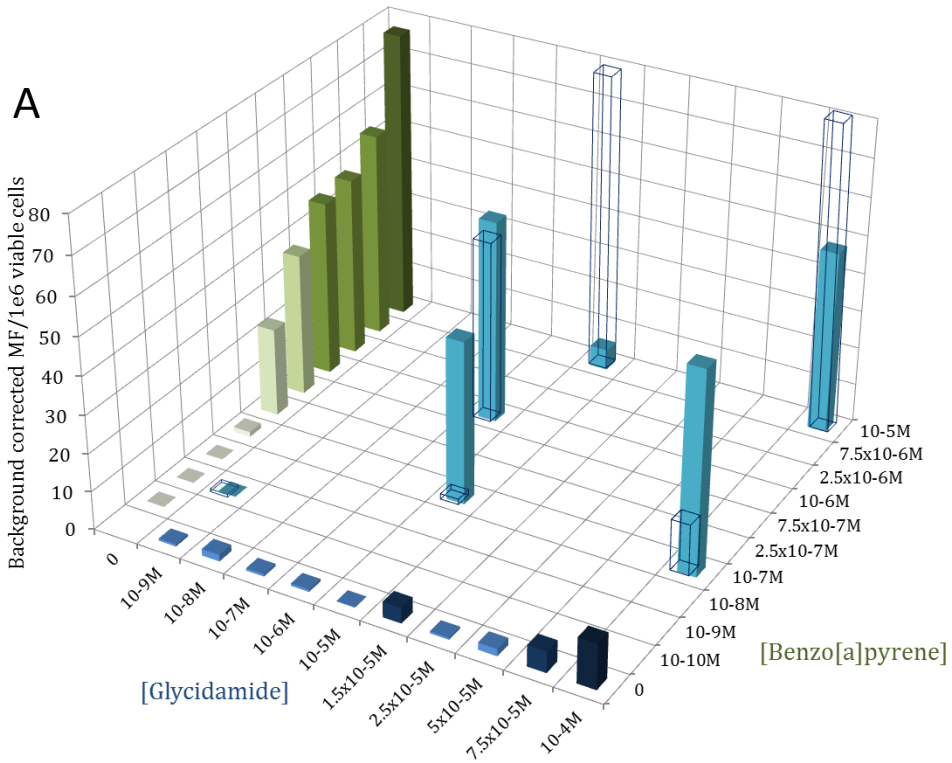
20
10
0
*
10-9M
10-8M
10-7M
10-6M
10-5M
1.5x10-5M
2.5x10-5M
5x10-5M
7.5x10-5M
10-4M
[GA]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Background corrected MF/1e6 viable cells
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60
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10-9M
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10-5M
[Glycidamide]
1.5x10-5M
2.5x10-5M
5x10-5M
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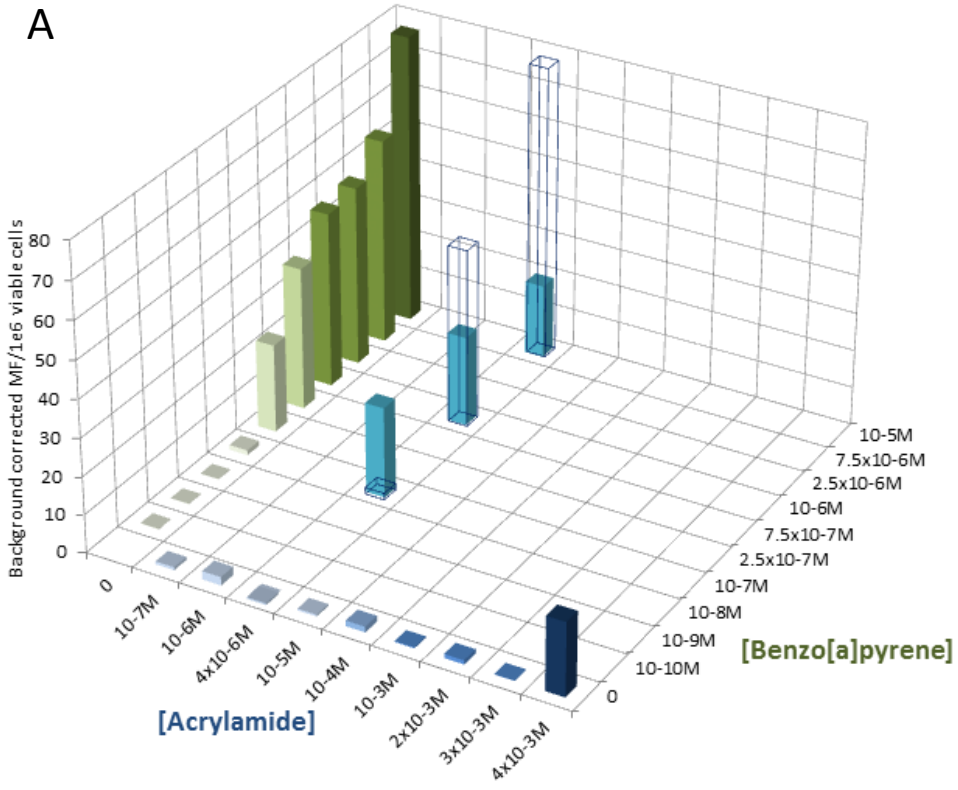
[Benzo[a]pyrene]

A


Background corrected MF/1e6 viable cells
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10-4M
10-3M
2x10-3M
3x10-3M
4x10-3M
[Acrylamide]
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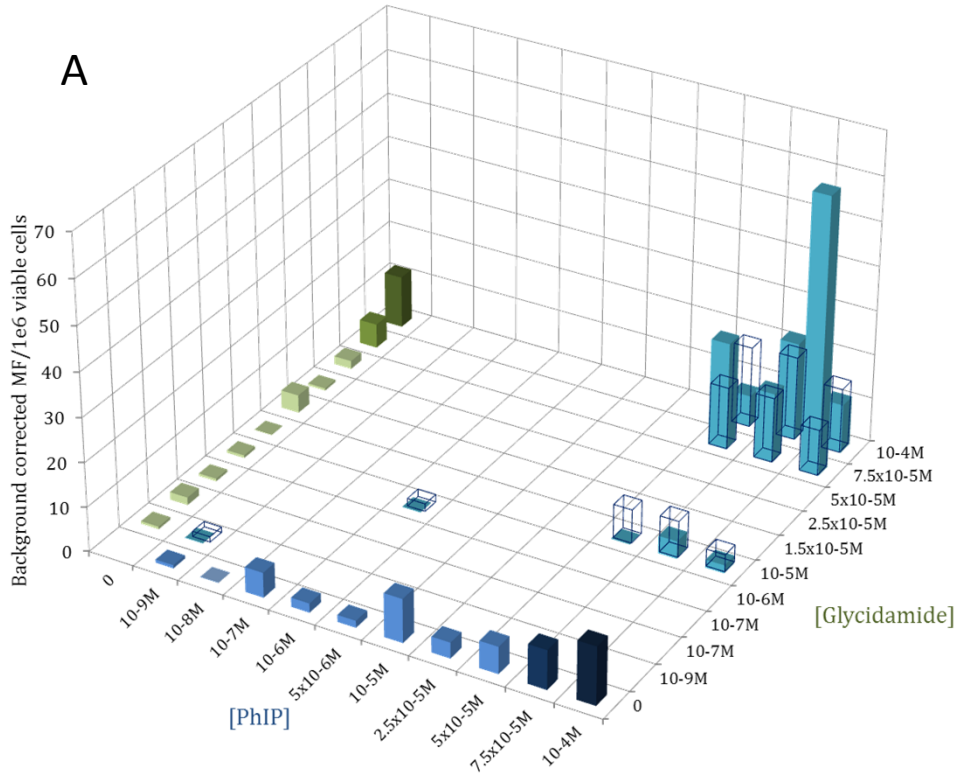
A
Background corrected MF/1e6 viable cells
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5x10-6M
10-5M
2.5x10-5M
5x10-5M
7.5x10-5M
10-4M
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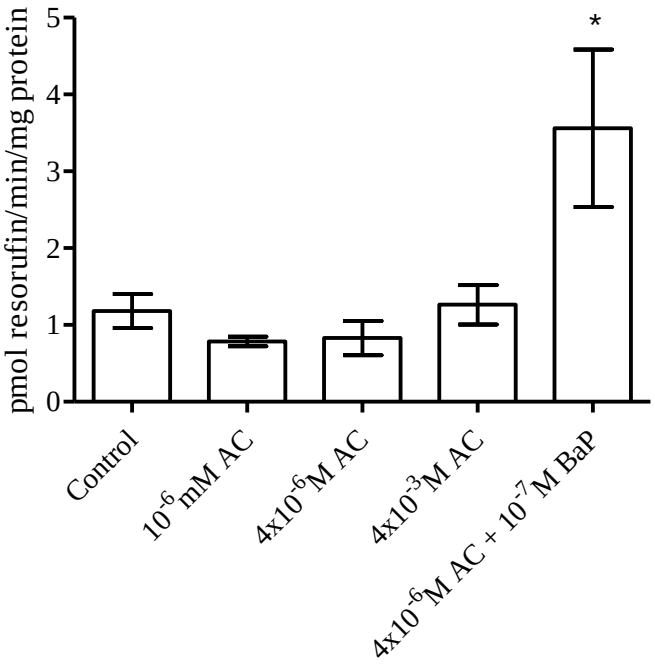
pmol resorufin/min/mg protein
5
4
3
2
1
0
*
Control
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4x10-6 M AC
4x10-3 M AC
4x10-6 M AC + 10-7 M BaP