Toxicology Research

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Interleukin-6 promotes dietary carcinogen-induced DNA damage in colorectal cancer cells.

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Running title: IL6 promotes carcinogen-induced DNA damage.

Word count: 2820 (excluding abstract, references and figure legends)

Number of figures: 6

Number of tables: 0

Key words: Inflammation, micronucleus assay, cytochrome P450, colorectal cancer, dietary carcinogen, DNA damage, 3D cell culture.

Conflict of Interest: None declared

Abstract

Colorectal cancer (CRC) is the third most common cancer worldwide with 80% of cases being sporadic, arising following a series of environment-induced gene mutations. DNA damaging pro-carcinogens such as benzo[a]pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) contained in red or processed meats are a potential risk factor for disease. These dietary pro-carcinogens require metabolic activation to their genotoxic agents by cytochrome P450 (CYP) family 1 enzymes. We have previously demonstrated that the pro-inflammatory cytokine interleukin-6 (IL6) promotes *CYP1B1* expression in CRC cells grown as 2D monolayers and that these two proteins are overexpressed in malignant tissue resected from CRC patients, indicating that inflammation influences metabolic competency in CRC cells. To determine whether IL6 can influence BaP and PhIP activation, we investigated IL6 effect on BaP- and PhIP-induced DNA damage in CRC cell lines grown as 2D monolayers and as 3D spheroids using the *in vitro* micronucleus (MN) assay. We also investigated the involvement of p53 and CYPs in the observed effects. MN formation was increased dose-dependently following treatment with BaP and PhIP while pre-treatment with IL6 further enhanced DNA damage. We confirmed that IL6-mediated effects were not caused by p53 expression changes but rather by *CYP1B1* expression induction through miR27b downregulation. Taken together, these data demonstrate that inflammatory cytokines can promote dietary pro-carcinogen activation and DNA damage in CRC cells.

Introduction

As the colon is part of the digestive system, it is particularly susceptible to carcinogens present in the diet. Epidemiological studies have reported a significant correlation between high consumption of red and processed meats, known sources of dietary carcinogens, to increased incidence of CRC [1].

Commonly occurring dietary carcinogens include benzo[a]pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [2–4]. BaP is a five-ring polycyclic aromatic hydrocarbon generated during the combustion of organic compounds and is commonly found in motor fumes, cigarette smoke and cooked meats [5,6]. It is thought to be involved in tobacco- and diet-associated cancers due to its mutagenic potential. PhIP is the most abundant heterocyclic amine found primarily in meats cooked at high temperatures, particularly fish, chicken and beef [7]. Studies have found correlations between meat consumption, PhIP intake and colon, breast and prostate cancers [1,8–10], and PhIP-DNA adducts have been detected in these tissues [11–13], supporting its role as a mutagens in these tissue types.

Like many carcinogens, BaP and PhIP are activated into their genotoxic derivatives, 7,8-diol-9,10-epoxy BaP and N-hydroxy PhIP respectively, by CYP1 A1, A2 and B1 enzymes [6,14,15]. The activated molecules covalently bind DNA disrupting the double-helical structure resulting in DNA damage including double-strand breaks, deletions and points mutations [6,16–19].

CYP1B1 and CYP1A1 are found extra-hepatically and are expressed in CRC tissue [20–22], suggesting a potential for *in situ* metabolism and increased susceptibility to dietary carcinogen-induced mutations. However, mechanisms that regulate carcinogen activation in the colon are not yet well understood.

Overexpression of pro-inflammatory cytokine interleukin-6 (IL6) occurs at the tumour site in CRC patients [23,24] and we recently determined that *CYP1B1* expression was epigenetically regulated by IL6 through miR27b in CRC cells grown as 2D monolayers [20]. Given our previous findings, IL6 may be involved in promoting activation of dietary pro-carcinogens in colonic cells.

In vitro techniques commonly use cells cultured as 2D monolayers. However, when cultured in this manner, cells are flattened onto the culture surface thus changing their structure, and parameters such as cell-to-cell interaction and tissue architecture are lost. *In vitro* 3D cell culture systems, in which cells are grown as spheroids, constitute a better model of *in vivo* tissue without the use of animal models. Previous studies comparing 2D and 3D cell culture systems have shown 3D cell morphology and growth rate more accurately represent in vivo tumours [25]. Thus, 3D cell culture has become increasingly popular replacing standard cell culture techniques as a means of increasing *in vivo* relevance of *in vitro* experimental results.

In the current study, we have adapted current *in vitro* techniques for use with 3D cultures and have compared results with that obtained with cells grown as 2D cultures. We have used this novel technique to investigate the mechanisms involved in diet-associated CRC by studying the effect of IL6 on BaP- and PhIP-induced DNA damage as well as underlying mechanisms for observed effects using *in vitro* models. Exploring these mechanisms is important to further understand the role of IL6 in diet-associated colorectal carcinogenesis and could potentially identify a novel regulator of dietary carcinogen activation.

Materials and methods

Cell culture

The human colorectal adenocarcinoma cell lines HCT116 and SW480 were obtained from ATCC (LGC Prochem, Middlesex, UK). HCT116 p53-/- cells were kindly provided by Professor Bert Vogelstein (John Hopkins University, Baltimore, MD, USA). Cells were routinely cultured in RPMI1640 medium (GIBCO, Life technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 100units/ml penicillin, 100μg/ml streptomycin and 2mM *L*-glutamine (GIBCO, Life technologies). All cells were incubated at 37°C in a humidified incubator (5% CO_2). Cells between passages 3-7 were used for experiments.

For 3D cell culture, cells were seeded at a density of 5 x 10^5 cells/well in a 24-well Algimatrix system (Invitrogen, Life technologies) according to the manufacturer's protocol. Cells were monitored and culture medium was routinely changed. Spheroids cultured for 7-10 days were used in all experiments. To isolate spheroids from the matrix, matrix-dissolving buffer (Invitrogen, Life technologies) was used according to manufacturer's protocol.

Cell treatments

Prior to treatment, HCT116 and SW480 were maintained in culture medium supplemented with 5% dextran-coated charcoal-stripped FBS for at least 72 hours. Cells were seeded at a density of 1x10^5 cells per well of a 6-well plate (for 2D cell culture). HCT116 and SW480 do not express IL6 so human recombinant IL6 (HumanKine, Sigma-Aldrich, Dorset, UK) dissolved in PBS

containing 0.1% human serum albumin (Sigma-Aldrich) was added to the cells for 24 and 48 hours at doses of 0-5000pg/ml (chosen within the range secreted by stromal cells in the colon [26]).

For treatment with dietary carcinogens, cells were pre-treated with IL6 as described above, washed with PBS and treated with a dose-range of BaP (0-10μM, Sigma-Aldrich) and PhIP (0-100 μM, Toronto Research Chemicals Inc., Toronto, Canada). Both chemicals were dissolved in DMSO and final vehicle control concentration of 0.2% was used.

Cytotoxicity and micronucleus assay

Micronucleus (MN) assay was performed according to OECD guidelines with modifications. Briefly, cells were seeded at a density of $5x10^4$ cells per well of a 24-well plate for 2D cell culture. Spheroids grown for 10 days were used for 3D culture. Cells were treated with IL6, BaP or PhIP as detailed previously, washed with PBS and cultured for a further 72 hours prior to harvest. Etoposide (Sigma-Aldrich) was used as a positive control in all assays at a concentration of 125nM. Cells were then harvested, resuspended in culture medium containing 2% pluronic (GIBCO, Life technologies) and cytotoxicity was determined by counting cells in a haemocytometer with TrypanBlue exclusion (GIBCO, Life technologies). Cells were fixed with 100% methanol onto microscope slides at a density of $2x10^4$ cells per slide and stained for 60 seconds with acridine orange (0.1mg/ml dissolved in PBS, Sigma-Aldrich). Frequency of MN was scored blind in 1000 cells per sample and three biological replicates were performed per treatment.

RNA extraction

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Life technologies) according to the manufacturer's protocol. RNA extracts were quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280nm and 260/230nm ratios. Extracts were stored at -80°C until used.

Reverse transcription and qPCR

Reverse transcription and qPCR were performed as previously described [20]. Briefly, for mRNA reverse transcription, Superscript II reverse transcription kit was used (Invitrogen, Life technologies) and miRNA reverse transcription kit was used for miRNA expression (Applied Biosystems, Life technologies). QPCR was performed using pre-designed expression assays (Taqman, Applied Biosystems, Life technologies) for *CYP1A1* (Hs01054797_g1), *CYP1B1* (Hs00164383_m1), *p53* (Hs01034249_m1), *GAPDH* (Hs99999905_m1), U6 (001973) and miR27b (000409). FAST PCR master mix was used according to the manufacturer's protocol (Taqman, Applied Biosystems, Life technologies).

Statistical analysis

Data were obtained from measurements made in at least three biological replicates and presented as a mean ± standard error (SEM). Significant differences ($p<0.05$) were determined using Student's t-test, one-way analysis of variance (ANOVA) followed by a Dunnett post-test. Pearson's product-moment correlation coefficient test was used for correlation analysis (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA).

Results

1. IL6 effect on chemical-induced DNA damage by food-derived pro-carcinogens BaP and PhIP

The *in vitro* MN assay is a commonly used toxicological test for detecting genotoxic potential of compounds due to its simplicity of scoring, accuracy and adaptability to different cell types. More importantly, studies in humans have shown strong associations between micronuclei frequency and cancer risk [27]. Formation of micronuclei, i.e. small membrane-bound DNA fragments in the cytoplasm, occurs during cell division when a whole chromosome or a chromosomal fragment is not incorporated into the nucleus of one of the daughter cells. Standard MN assays utilise immature blood cells, but non-standard versions of the assay have been developed using different cell types including epithelial cells. Here, we have adapted the assay for use with human colorectal adenocarcinoma epithelial cell lines grown as 2D and 3D cultures.

Cytotoxicity

Cytotoxicity was measured following IL6 and carcinogen treatment to ensure cell viability post-treatment. While some toxicity (around 30% drop in cell survival) was observed particularly with IL6 + BaP treatment in both cell lines, these differences were not statistically significant compared to respective controls (Fig. 1).

Genotoxicity

A dose-dependent increase in BaP- and PhIP-induced MN frequency was observed in HCT116 and SW480 cell lines cultured as 2D monolayers. Interestingly, pre-treatment with IL6 significantly enhanced the pro-

carcinogen-induced DNA damage while treatment with IL6 on its own had no effect (Fig. 2). Etoposide, a topoisomerase II inhibitor and potent inducer of MN formation, was used as a positive control and does not require activation to induce DNA damage. Pre-treatment with IL6 did not enhance etoposide-induced DNA damage suggesting that IL6 may have an effect on the activation pathway of the pro-carcinogens rather than on MN formation itself.

3D cell culture

The assay was repeated using HCT116 cells grown as 3D spheroids. In general, results (Fig. 3) were in agreement with that found in 2D : no significant cytotoxicity was observed and pre-treatment with IL6 enhanced BaP and PhIP induced DNA damage. However, the increase in DNA damage observed with IL6 pre-treatment was not as pronounced in 3D culture compared to 2D: MN frequency was increased by 1.5 fold in 3D culture with IL6 pre-treatment while in 2D culture it was increased by 2-3 fold (Fig. 2). Taken together, these data demonstrate that presence of IL6 in colon epithelial cells along with food-derived pro-carcinogen can enhance induction of DNA damage. We therefore investigated the mechanism by which IL6 exerts this effect. IL6 is a pleiotropic cytokine with a multitude of functions; it has previously been shown to regulate *p53* gene expression [28] and *CYP1B1* expression [20], thus we investigated whether these two pathways were responsible for IL6 mediated effects.

2. Mechanisms underlying IL6 effects

P53 involvement

The tumour suppressor protein p53 induces cell cycle arrest upon DNA damage recognition, activates DNA repair pathways or induces apoptosis in the compromised cell; p53 is thus crucial to maintain genomic stability. IL6 has been reported to induce downregulation of *p53* gene expression by promoting methylation of its promoter region [28]. Both of the cell lines used in this study have p53 activity. To test whether inactivation of p53 may be responsible for the IL6 effect observed, we performed a MN assay on HCT116 p53-/- cells obtained from Professor Vogelstein's laboratory (John Hopkins University, Baltimore, MD, USA) and looked at the effect of IL6 treatment on *p53* gene expression by qPCR. No significant downregulation of *p53* gene expression was observed at the dose of IL6 used in this study (Fig. 4 A, B and C). Previous studies used supraphysiological doses of IL6 (10ng/ml [28]) and therefore, the dose used here (physiological levels) are likely to be too low to inhibit *p53* expression. In addition, MN frequency was not increased in the p53-null HCT116 cells when compared to wild-type HCT116, rather total number of MN was decreased (Fig. 4 D and E). This demonstrates that the observed effect of IL6 on MN induction by BaP and PhIP is not caused by downregulation of *p53*, further suggesting that IL6 may affect the activation pathway of the pro-carcinogens *via* induction of *CYP1B1* expression as previously described [20].

CYP450 involvement

Previous studies in this laboratory have demonstrated that IL6 can regulate *CYP1B1* expression *via* miR27b downregulation but not *CYP1A1* in CRC cells grown as 2D monolayers [20], indicating that IL6 may be associated with a change in metabolic competency. In the current study, we examined the effect

of IL6 treatment on *CYP450* expression in CRC cells grown as 3D spheroids to confirm our previous findings. As expected, treatment with IL6 did not alter *CYP1A1* expression (Fig. 5 A). Upregulation of *CYP1B1* in both HCT116 and SW480 3D spheroids following IL6 treatment was observed along with downregulation of miR27b (Fig. 5 B and C), thus confirming our previous observations in 2D culture [20]. Therefore IL6 regulates *CYP1B1* expression by downregulating miR27b.

Furthermore, we investigated the effect of 24-48 hour pre-treatment with IL6 followed by removal of the IL6 media then 24 hour BaP or PhIP treatment on *CYP1A1*, *CYP1B1* and miR27b expression. However, we found no significant changes with IL6 pre-treatment followed by BaP or PhIP compared to BaP or PhIP treatment alone (Fig. 6), likely due to the fact that IL6 is removed prior to BaP and PhIP treatment. Therefore, *CYP1B1* induction is not sustained following IL6 removal. These data suggest that the initial induction of *CYP1B1* expression (in the first 24 to 48 hours) by IL6 treatment is responsible for increased carcinogen activation and DNA damage.

BaP (but not PhIP) is a known inducer of the AhR pathway that controls *CYP1A1* and *CYP1B1* expression and here as expected, BaP significantly induced expression of both *CYP1B1* and *CYP1A1*. Furthermore, miR27b expression is not altered following BaP and PhIP treatment, indicating that unlike IL6, BaP regulates *CYP1B1* expression *via* the AhR pathway and not miR27b.

Discussion

In the current study, IL6 was shown for the first time to promote DNA damage induced by BaP and PhIP, two carcinogens present in meats cooked at high

temperatures. Umannová *et al.* previously reported that TNFα, another pro-inflammatory cytokine, increased BaP-induced genotoxic damage in alveolar epithelial type II cells [29], suggesting that other inflammatory cytokines may have a similar effect, however specific mechanisms were not presented. TNFα is a known inducer of IL6 and therefore, IL6 may have been involved in the effect reported by Umammova *et al.* (25). Here, we suggest that IL6 promotes BaP- and PhIP-induced genotoxicity through induction of *CYP1B1* expression.

Previous studies on IL6 regulation of CYP450 enzymes present conflicting reports. Generally IL6 is thought to have an inhibitory effect in hepatic cells [30–33], however a few studies in other cell types have shown increased expression of CYP450 in response to IL6 [20,34–36], which are in agreement with the data presented here in CRC cells grown as 3D spheroids. As previously shown in 2D cultures [20], we found that IL6 downregulated miR27b expression resulting in increased *CYP1B1* expression in 3D cultured CRC cells. Furthermore, we also determine that IL6 mediated regulation of *CYP1B1* was not sustained following IL6 removal and determined that the higher levels of CYP1B1 in the cells caused by IL6 pre-treatment were likely to be responsible for the increased activation of BaP and PhIP. Moreover, we determined that BaP and PhIP do not regulate miR27b expression.

BaP and PhIP are also prevalent in other environmental factors such as pollution or smoking. In addition, CYP1B1 can also activate other environmental carcinogens [37–41], and smokers have been shown to have higher plasma levels of IL6 compared to non-smokers [42]. Thus this novel IL6-mediated carcinogen activation pathway could also be important in other

environment-associated cancers such as lung, and preventing IL6-mediated expression of CYP450s could potentially be an effective disease prevention strategy. Regular intake of NSAIDs has been associated with lower cancer risk including CRC, breast and lung, however the specific underlying mechanisms remain unclear [43–45] and prevention of IL6-mediated induction of *CYP1B1* expression may be responsible, at least in part, for this effect.

CYP450 enzymes are also involved in metabolising a wide array of drugs, thus identifying mechanisms of their regulation in tumours could have significant implications in cancer therapies. High levels of IL6 at the tumour site has been associated with multiple drug resistance in a variety of cancer types [46,47]. IL6-mediated induction of local CYP450 expression could be involved in this effect as these enzymes could be inactivating chemotherapeutic drugs, thus administrating selected drugs that are not inactivated by these enzymes or combining drugs with an anti-IL6 adjuvant therapy could potentially attenuate drug resistance.

The IL6-mediated effects were observed in different *in vitro* culture systems (2D and 3D) as well as in two distinct CRC cell lines (HCT116 and SW480), thus providing further validation of the current findings. When comparing different cell culture methods, we observed that IL6-mediated effects on promoting BaP- and PhIP-induced DNA damage were not as pronounced in 3D cultures as compared to 2D cultures. Furthermore, for SW480 cells, higher doses of IL6 were required in 3D culture in order to reproduce responses observed in 2D culture. It has generally been shown that a decrease in drug sensitivity is observed in cells cultured in 3D compared to 2D [48,49] likely due to the differences in level of exposure caused by the architecture of the

spheroid, thus 3D culture better recapitulates *in vivo* responses.

Conclusion

In the current study, we investigated the effect of IL6 on DNA damage caused by dietary pro-carcinogens BaP and PhIP in CRC cells grown as 2D monolayers and 3D spheroids. Pre-treatment with IL6 enhanced BaP- and PhIP-induced DNA damage by promoting the activation of the carcinogens through *CYP1B1* expression induction. Increased levels of CYP1B1 in the cell along with presence of dietary carcinogens would lead to increased quantities of genotoxic metabolites, thus resulting in DNA damage. Discovery of this novel pathway provides further understanding of the mechanisms regulating dietary carcinogen activation in colonic cells and provides a mechanistic basis for the established chemopreventive activity of nonsteroidal anti-inflammatory drugs in chemical-mediated colorectal carcinogenesis [50].

Acknowledgements

The work was supported by funding from the United Kingdom Medical Research Council.

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Figure legends

Fig. 1 Cytotoxicity of 2D HCT116 and SW480 induced by BaP and PhIP. HCT116 (A, B) and SW480 (C, D) cells grown as monolayers were pre-treated with IL6 for 48 hours and 24 hours respectively, followed by 24 hour treatment with BaP or PhIP. Cells were harvested 72 hours post-treatment. Cytotoxicity following treatment expressed as % of cell survival. Statistically significant differences were calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3).

Fig. 2 DNA damage measured by MN frequency in 2D HCT116 and SW480 induced by BaP and PhIP. HCT116 (A, B) and SW480 (C, D) cells grown as 2D monolayers were pre-treated with IL6 for 24 hours followed by 24 hour treatment with BaP or PhIP. Cells were harvested 72 hours post-treatment. Etoposide was used as a positive control. Micronuclei (MN) frequency per 1000 cells was measured following treatment. Statistically significant differences are shown for comparisons between carcinogen treated *vs* IL6 pre-treated samples (***p<0.001, **p<0.01, *p<0.05), IL6 alone *vs* IL6 pre-treated and carcinogen treated (†††p<0.001, ††p<0.01, †p<0.05) and vehicle *vs* carcinogen treated (‡‡‡p<0.001, ‡‡p<0.01, ‡p<0.05). Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3).

Fig. 3 Cytotoxicity and DNA damage in HCT116 grown as 3D spheroids. Cells grown as 3D spheroids on Algimatrix were pre-treated with IL6 for 24 hours followed by 24 hour treatment with BaP or PhIP. Cells were harvested

72 hours post-treatment. Etoposide was used as a positive control. (A) Cytotoxicity following treatment expressed as % of cell survival. (B) Micronuclei frequency per 1000 cells following treatment. Statistically significant differences are shown for comparisons between carcinogen treated *vs* IL6 pre-treated samples (*p<0.05),. Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3).

Fig. 4 Involvement of p53 in IL6-mediated induction of BaP- or PhIP-mediated DNA damage. HCT116 and SW480 cells grown as 2D monolayers (A, B) and 3D spheroids (C) were treated with IL6 and *p53* expression was measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are show relative to control. (D-E) HCT116 p53-/- and HCT116 wild-type (WT) cells were grown as monolayers and treated for 24 hours with BaP or PhIP. Cells were taken 72 hours post-treatment and micronuclei frequency per 1000 cells were determined following treatment. Significance was calculated using one-way ANOVA with Dunnett post-test comparing treated group to vehicle control (GraphPad Prism 5, ***p<0.001). Error bars represent the SEM for independent cultures (n=3).

Fig. 5 IL6 effect on *CYP1A1, CYP1B1* and miR27b expression in 3D cultures. HCT116 and SW480 cells grown as 3D spheroids were treated with 0, 1000 or 5000pg/ml IL6 for 24 hours. *CYP1A1* (A), *CYP1B1* (B) and *miR27b* (C) expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* for gene expression or U6 RNA for miRNA expression and are shown relative to control. Significance was assessed using Student's

t-test comparing the treated group to vehicle control (GraphPad Prism 5, $^{**}p<0.01$, $^{*}p<0.05$). Error bars represent the SEM for independent cultures (n=3).

Fig. 6 *CYP1A1*, *CYP1B1* and miR27b expression following IL6 pre-treatment and BaP or PhIP treatment in 2D cultured HCT116 and SW480 cells. Cells grown as monolayers were pre-treated with 1000pg/ml IL6 for 24 hours (SW480) or 48 hours (HCT116) followed by 24 hour treatment with BaP or PhIP. *CYP1A1* (A, C), *CYP1B1* (B, D) and *miR27b* (E, F) expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* for gene expression or U6 RNA for miRNA expression and are shown relative to control. Statistically significant differences were calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5) and are shown for comparisons between vehicle *vs* carcinogen treated samples ($^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$) and IL6 alone *vs* IL6 pre-treated and carcinogen treated ($\dagger\dagger\dagger p<0.001$, $\dagger\dagger p<0.01$, $\dagger p<0.05$). Error bars represent the SEM for independent cultures (n=3).

Figures

Fig. 1

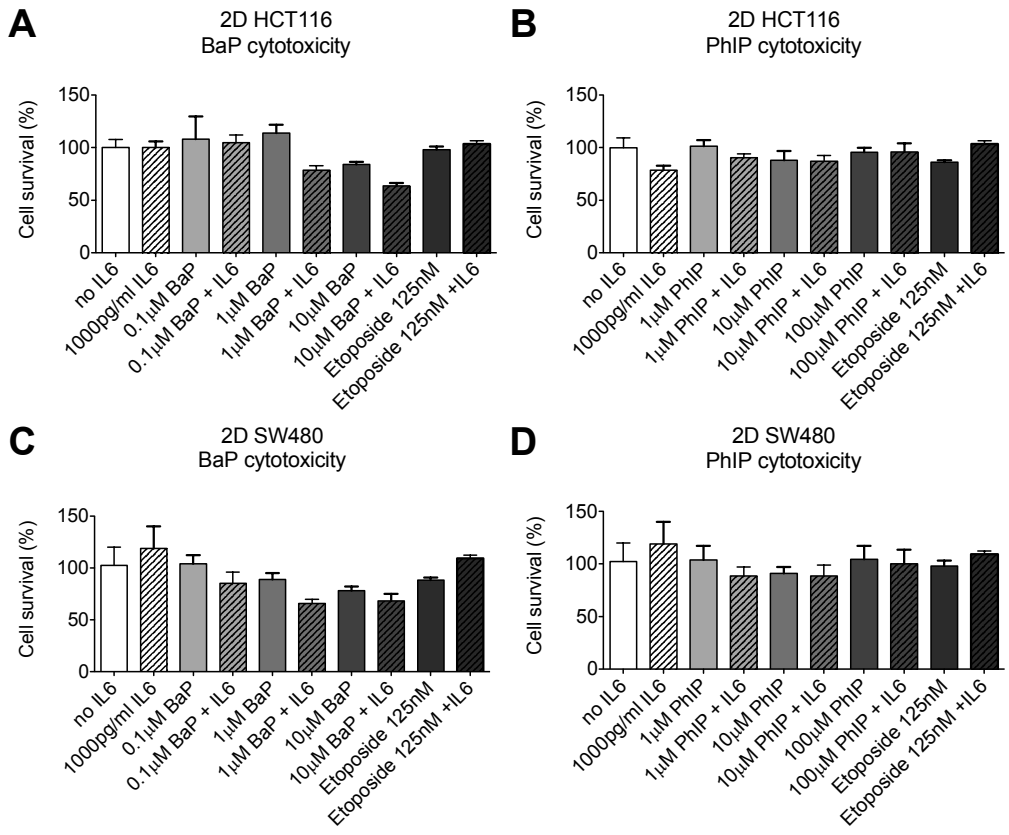

Fig. 2

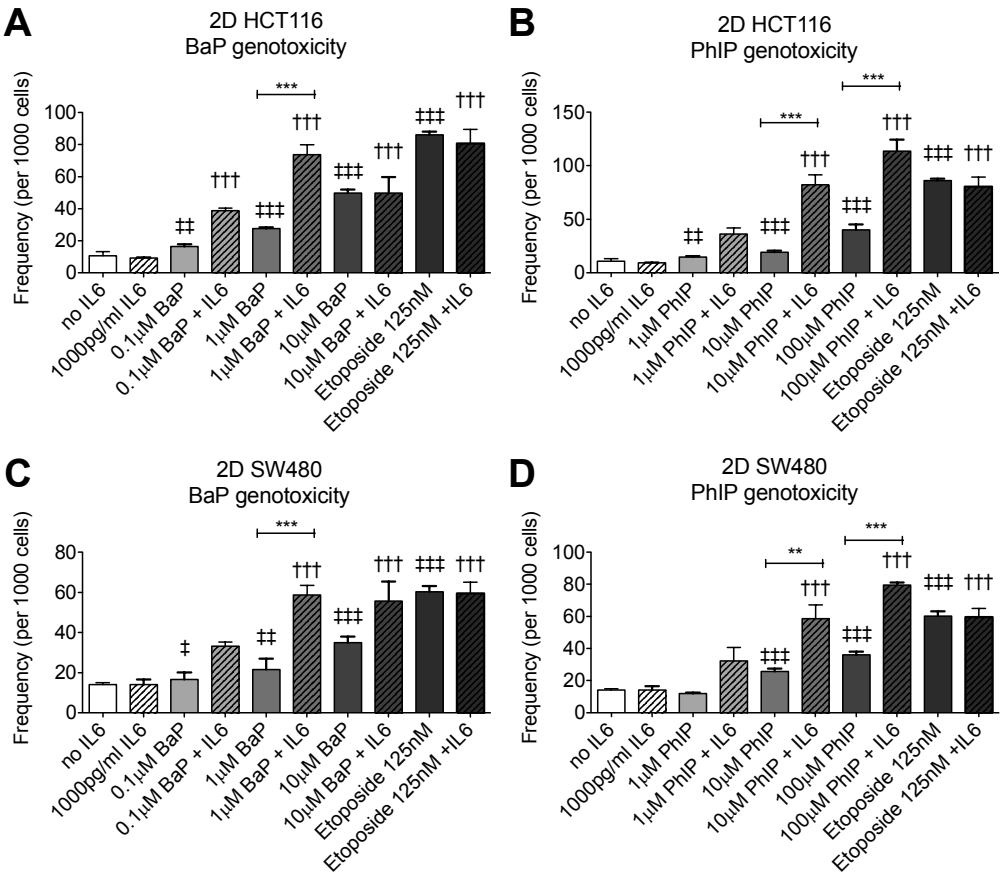

Fig. 3

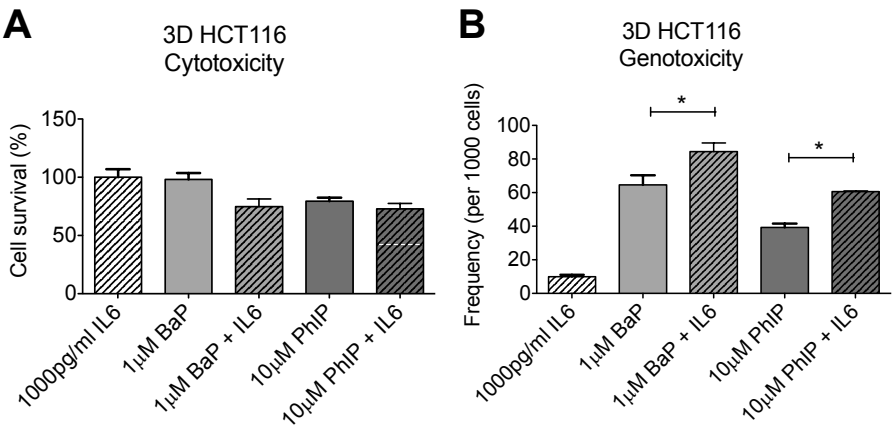

Fig. 4

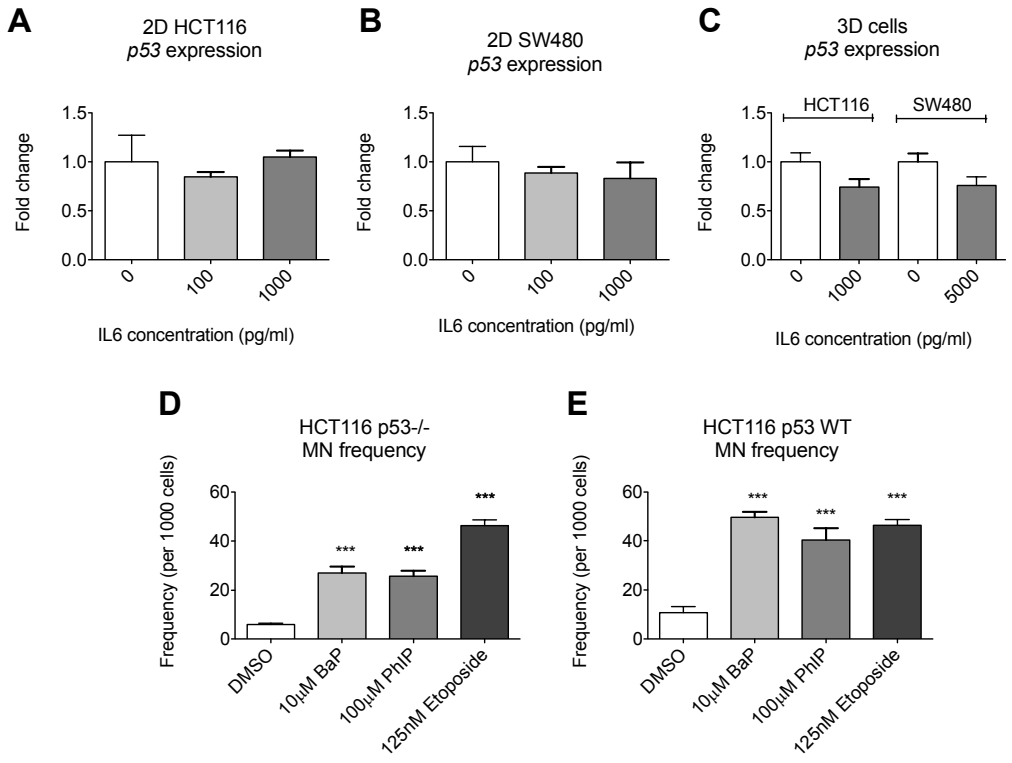

Fig. 5

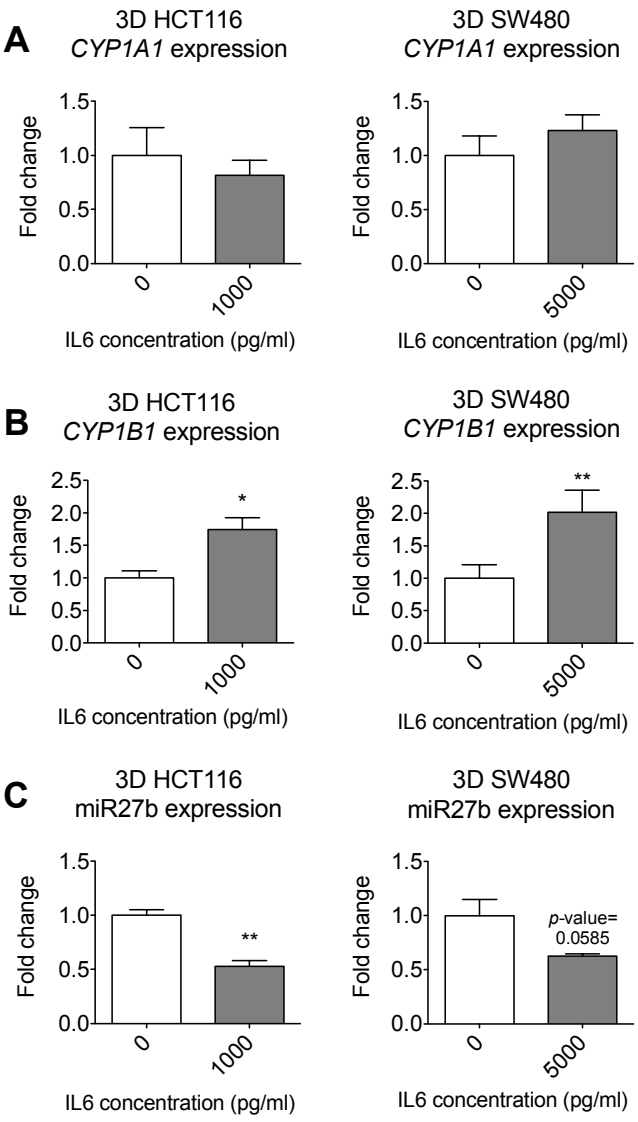

Fig. 6

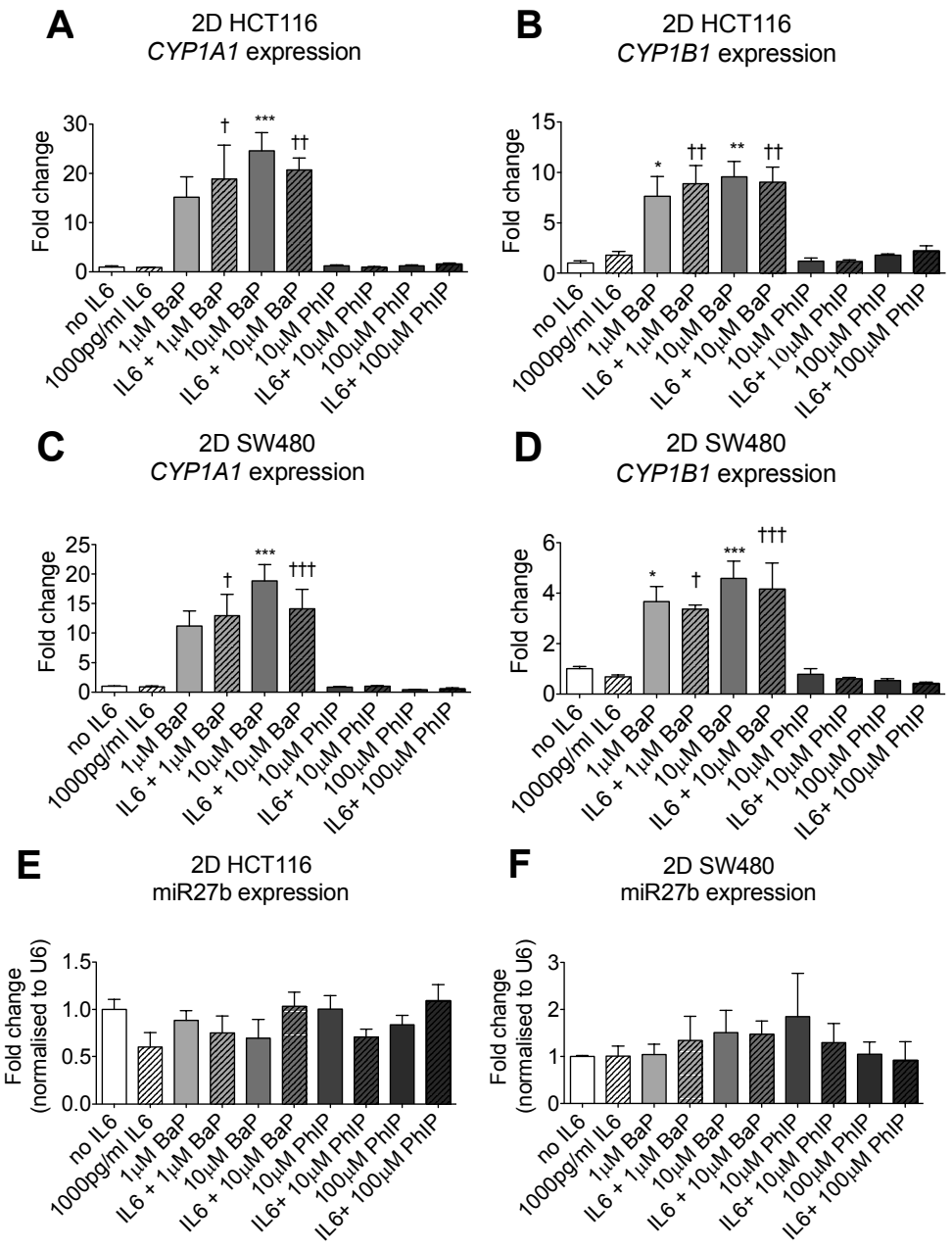

Table of contents entry:

Pro-inflammatory cytokine interleukin-6 promotes dietary carcinogen-mediated DNA damage in 2D and 3D cultured cells by inducing *CYP1B1* expression through miR27b downregulation.