Interleukin-6 selectively induces drug metabolism to potentiate the genotoxicity of dietary carcinogens in mammary cells

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Abstract
Breast cancer is the most commonly diagnosed malignancy in females, the etiology being multifactorial and includes the role of lifestyle exposure to DNA-damaging chemicals such as dietary carcinogens benzo (a) pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP). Both compounds require cytochrome P450 (CYP)-mediated metabolic activation to DNA-damaging species, and both induce transcriptional responses through the nuclear receptors Aryl hydrocarbon receptor (AhR) and estrogen receptor α (ERα). BaP and PhIP are mammary carcinogens in rodents. Clinically, circulating IL-6 expression is linked with poor prognosis of cancer and 35% of the deaths in breast cancer are linked with inflammation. The objective of this work was to investigate the molecular toxicology and local activation of BaP and PhIP in the presence of IL-6. Our laboratory has previously reported that miR27b can regulate CYP1B1 expression in colorectal cells, here we have investigated if this mechanism is working in mammary cell models, MCF-7 and MDA-MB-231 cells. Treatment (24 h) of cells with BaP (10 nM-10 µM) and PhIP (100 nM-100 µM) significantly induced genetic damage (micronuclei formation) in a dose-dependent manner in both cell lines. This effect was potentiated in the presence of human IL-6 at concentrations reported to be expressed in clinical breast cancer. On its own, IL-6 treatment failed to induce micronuclei frequency above the control levels in these cells. Compared to BaP or PhIP treatment alone, IL-6 plus BaP or PhIP selectively induced CYP1B1 significantly in both cell lines. Additionally, miR27b expression was downregulated by IL-6 treatments and transfection with miR27b inhibitor confirmed that miR27b is a regulator of CYP1B1 in both cell lines. These data show that BaP- and PhIP-induced DNA damage in mammary cells is potentiated by the inflammatory cytokine IL-6 and that inflammation-induced CYP expression, specifically CYP1B1 via miR27b, is responsible for this effect.

Keywords Benzo(a)pyrene · 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine · IL-6 · Inflammation · miRNA · Human mammary cells · Genotoxicity

Introduction

Epidemiological studies suggest that chronic inflammation increases the susceptibility of individuals to various types of cancer and is linked with 15–20% of all deaths from cancer worldwide (Bray et al. 2004; Mantovani et al. 2008). IL-6 is one of the most consistently up-regulated inflammatory mediators in breast tumors and is linked with malignant transformation of inflammatory breast cancer (IBC), an aggressive form of breast cancer (Zhang and Adachi 1999; Bachelot et al. 2003; Sanguinetti et al. 2015). The role of cytokines and particularly IL-6 to increase the proliferation, survival, epithelial–mesenchymal transition, invasion, and metastasis has been reported (Zhang and Adachi 1999; Fouad et al. 2014), however, its potential role in the early stages of carcinogenesis is less well understood. Oxidative stress, impairment of p53-dependent protective responses and modulation of various signaling pathways are linked with mutations involved in carcinogenesis associated with chronic inflammation and upregulation of cytokines (Lu et al. 2006). IL-6 can also affect the expression of specific CYP450s that can further influence carcinogenesis through altered bio-activation of various environmental carcinogens (Muntane-Relat et al. 1995).
In the main, studies on the effect of infection and inflammation on xenobiotic metabolism and activation report the suppression of CYP450s by cytokines such as IL-6 in liver and hepatocytes (Deloria et al. 1985; Gooderham and Mannering 1986; Morgan 1989; Muntane-Relat et al. 1995; Renton 2001, 2004; Aitken et al. 2006), however, recent studies in non-hepatic cell lines have demonstrated up-regulation of selective CYPs by IL-6 (Kurzawski et al. 2012; Patel et al. 2014; Patel and Gooderham 2015a, b), suggesting variation in response in a tissue-specific manner.

As much as 90–95% of cancers are reported to be preventable and have their origin from environment and lifestyle (Anand et al. 2008). Almost 25–30% of the cancer-related deaths are ascribed to tobacco, while 30–35% are linked to diet (Gooderham et al. 1996; Willett 2000; Barrett et al. 2003). Increased consumption of red meat and exposure to smoke in western lifestyle is linked with increased incidence of breast cancer (Zhu et al. 2003; Linos et al. 2007; Wu et al. 2009; Ronco et al. 2011). Benzo(a)pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) are commonly occurring environmental carcinogens (Sinha and Rothman 1999; Gooderham et al. 2002; Zheng and Lee 2009). BaP is present in tobacco smoke, pollution and in grilled food (Davis 1968; Kazerouni et al. 2001). PhIP is the most abundant heterocyclic amine in cooked meat (Zheng and Lee 2009; Felton et al. 1991; Lynch et al. 1992; Murray et al. 1993; Gooderham et al. 1997). BaP and PhIP are pro-carcinogens and are activated by CYP1 family A1, A2 and B1 enzymes into their genotoxic derivatives that bind covalently with DNA, disrupting the double helical structure leading to DNA damage (Strom and Michalopoulos 1982; Heussen et al. 1990; Boobis et al. 1994; Zhao et al. 1994; Kranendonk et al. 1998; Lynch et al. 1998; Gooderham et al. 2002, 2007). Activation of these CYP enzymes can increase the mutagenicity of various pro-carcinogens.

The regulation of CYP1 family enzymes is generally thought to be under control of inducible promoter regions, however, other epigenetic mechanisms are also reported to contribute to expression. In particular, microRNA (miRNA) has been shown to post-transcriptionally control numerous gene targets. MicroRNAs are a class of non-coding RNA that have powerful post-transcriptional gene expression regulation properties and have been found to be important contributors to the development of cancer. MiRNA are shown to regulate various genes including tumor suppressor and onco-genes that are involved in the progression of breast cancer (Bachour and Bennett 2011). Moreover, they can regulate various genes including tumor suppressor and onco-genes that are involved in the progression of breast cancer. MiRNA are shown to regulate various genes including tumor suppressor and onco-genes that are involved in the progression of breast cancer. MiRNA are shown to regulate various genes including tumor suppressor and onco-genes that are involved in the progression of breast cancer. MiRNA are shown to regulate various genes including tumor suppressor and onco-genes that are involved in the progression of breast cancer.

Methods

Cell culture and treatment

The human breast adenocarcinoma MCF-7 (ER-α+) and MDA-MB-231 (ER-α−) cell lines were purchased from ATCC (LGC Prochem, Middlesex, UK) and were grown in minimum essential medium (MEM) (GIBO, Life technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 µg/ml) and 2 mM L-glutamine. Cells were cultured routinely in 75 cm² flasks in a humidified incubator at 37 °C, 5% CO₂. Prior to treatment, cells (MCF-7 and MDA-MB-231) at a density of 25,000 cells/well in 24-well plates, were cultured in MEM supplemented with 5% dextran-coated charcoal-stripped FBS (Stripped media) for 72 h. Cells were treated with PhIP (0–100 µM, Toronto Research Chemicals Inc., Toronto, Canada) or BaP (0–10 µM), dissolved in dimethyl sulphoxide (DMSO) or IL-6 (0–20,000 pg/ml, Humankine, Sigma-Aldrich, Dorset, UK) dissolved in PBS with 0.1% human serum albumin (Sigma-Aldrich). In some experiments, cells were treated simultaneously with IL-6 and BaP or PhIP.

Cytotoxicity and micronucleus assay

Cytotoxicity and micronucleus (MN) assays were performed according to OECD guidelines adapted to MCF-7 and MDA-MB-231 cells. Briefly, cells were seeded at a density of 5 × 10⁴ cells per well in 24-well plate. Cells were treated with BaP or PhIP or IL-6 or in combination as described in the text. Following treatment with chemicals and harvesting (48 h), cells were trypsinised, the cell concentration adjusted to 2 × 10⁵ and re-suspended in serum-free R₀ (serum-free media) with 2% pluronic acid medium (GIBCO, Life technologies) and cytotoxicity was determined by counting cells in a haemocytometer with TrypanBlue exclusion (GIBCO, Life technologies). For the MN assay, cells were spread on a microscope slide using a cytospin. Cells at a density of 2 × 10³ cells per slide were fixed with 100% methanol and stained for 60 s with acridine orange (0.1 mg/ml dissolved in PBS, Sigma-Aldrich). Frequency of MN was scored in 2000 cells per sample and three biological replicates were performed per treatment. Etoposide (1.25 µM) was used as a positive control.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Following treatment, cells were lysed using TRIZOL reagent and chloroform (0.2 ml) was added to each sample and...
Cells were seeded at a density of 1 × 10^5 cells/well in 24-well plate and allowed to settle overnight in 10% FBS MEM medium (no penicillin/streptomycin). Following overnight incubation, media was replaced with 400 µl/well opti-MEM media (Gibco, Life Technologies), followed by the addition of 150 µl/well of Opti-MEM containing 2.5 µl of Lipofectamine 2000 reagent and 2.5 µl of 20 µM stock of miRNA inhibitor or miRIDIAN miRNA negative control (Dharmacon, UK). Transfected MCF-7 and MDA-MB-231 cells were incubated at 37 °C, 5% CO₂ for 24 h and 48 h, respectively, before harvesting RNA with Trizol reagent (Invitrogen).

**Statistical analysis**

The difference in treatments vs control was compared by one-way analysis of variance (ANOVA) followed by a Dunnett’s post-test. Data were obtained from measurements made in at least three independent cultures and presented as a mean ± standard error (SEM). Pearson’s correlation coefficient test was used for correlation analysis (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA).

**Results**

**Effect of BaP, PhIP and IL-6 on CYP1 family enzyme expression**

The expression of CYP1A1, CYP1A2 and CYP1B1 was inducible by BaP treatment in a dose-dependent manner; the magnitude of the induction was greatest for CYP1A1 and CYP1B1 in both cell lines (Table 1). In contrast, PhIP failed to induce expression of CYP1A1 but did induce the expression of CYP1A2 and CYP1B1 in both cell lines, albeit to a lesser extent than CYP induction by BaP.

Following 24 h treatment of MCF7 and MDA-MB-231 cell lines with IL-6 (0–20 ng/ml), little change in CYP1A1 and CYP1A2 expression was observed, however, a dose-dependent induction in CYP1B1 was seen in both cell lines (Fig. 1e and f). Compared to control, the lowest concentration of IL-6 that significantly induced CYP1B1 in both cell lines was 500 pg/ml (Fig. 1e and f). This is at the higher levels of circulating IL-6 in cancer patients, reported to be between 10 and 500 pg/ml (Blay et al. 1992; Goswami et al. 2013; Brouwers et al. 2015), but below the IL-6 levels reported to be present in breast tumor tissue (3–5 ng/g tumor) (Ueno et al. 2000; Knupfer et al. 2004; Knupfer and Preiss 2007). Thus, in subsequent co-culture experiments we used a maximum of 500 pg/ml IL-6. It is worth noting here that most reported mechanistic studies with mammary cancer cells have used a concentration of IL-6 in the ng/ml range (Yamashita et al. 1993; Hobisch et al. 2001; Knupfer and Preiss 2007; Oh et al. 2011; Deshmukh et al. 2015).

Whilst IL-6 is reported to down-regulate the expression of CYP1A1 and CYP1A2 in liver cells (Muntane-Relat et al. 1995), recent studies using colorectal cells have shown that IL-6 upregulates the transcription of CYP1B1 (Patel et al. 2007Archives of Toxicology (2019) 93:3005–3020

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**Ethoxyresorufin-O-deethylase**

Ethoxyresorufin-O-deethylase (EROD) assay was used as a measure of CYP1 family activity. Following treatment, 7-ethoxyresorufin (8 mM) was added to cells (2 × 10^6 in 1 ml of media) to give a final concentration of 8 µM. The plate was incubated at 37 °C for 90 min in a fluorescent plate reader (BMG POLARstar Galaxy Labtech, Ortenberg, Germany) and read at 10 min intervals at 37 °C at λ excitation (λex) 560 nm and λ emission (λem) 590 nm. A resorufin standard curve was run by adding stock solution to give final concentrations of 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 0.5 nM. Subsequently, total protein was extracted from cells collected in each assay and quantified. Enzyme activity was expressed as pmol resorufin formed per min per mg protein. Rat liver S9 fraction which expresses high levels of CYP was used as a positive control for the assay. Cells were collected and centrifuged at 1500 rpm and washed with serum-free media (Ro). The pellet was stored at − 80 °C. Immunoprecipitation (IP) lysis buffer (200 µl) was added, followed by vortexing, sonication (30 s), incubation on ice (30 min) and finally centrifugation to remove debris (10,000 rpm at 2–4 °C for 5 min). Bicinchoninic acid (BCA) assay (Pierce, ThermoScientific) was performed to determine protein concentration according to manufacturer’s instructions.

**Transfection with miRNA inhibitor**

Centrifuged 12,000 g (10 min, 2–8 °C). The upper aqueous phase was transferred to a fresh tube and 5 µg of RNase-free glycogen (as carrier to aqueous phase) and 0.5 ml isopropanol alcohol were added to precipitate RNA and incubated (37 °C, 10 min). Following incubation, lysates were centrifuged at 12,000 g (10 min, 2–8 °C). The gel-like pellet was washed with ethanol and re-dissolved in RNase-free water with heating (55–60 °C). Extracted RNA was quantified by UV spectroscopy (UV–Vis Nano-spectrophotometer, Implen, Essex, UK) and purity was assessed from 260/280 to 260/230 nm ratios. Reverse transcription (RT) of extracted RNA (100–500 ng) was completed according to manufacturer’s protocol (Invitrogen) and qPCR was performed using presynthesized Taqman gene expression assays and FAST PCR master mix (Taqman, Applied Biosystems, Life technologies) using a StepOnePlus fast real-time PCR system (Applied Biosystems, Life technologies). Target gene expression was normalized to GAPDH and quantified using the delta-Ct method (Livak and Schmittgen 2001).

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The studies reported here extend this observation to breast cancer cells.

**Can IL-6 augment BaP/PhIP-mediated CYP induction?**

In addition to their ability to induce the expression of the CYP1 family enzymes, both BaP and PhIP are activated to their genotoxic metabolites by CYP1A1, CYP1B1 and CYP1A2 enzymes. Since IL-6 treatment of MCF-7 and MDA-MB-231 cells also induced CYP1B1, we examined the effect of co-treatment of MCF-7 and MDA-MB-231 cells with IL-6 and BaP/PhIP. Co-treatments of IL-6 with BaP elevated CYP1B1 gene expression significantly above that induced by BaP alone in both in MCF-7 (Fig. 2c) and MDA-MB-231 (Fig. 3c), but there was no similar additional induction of CYP1A1 and CYP1A2 (Figs. 2 and 3) compared to BaP or PhIP alone, in either cell line.

CYP1 family enzyme activity can be assessed by monitoring ethoxyresorufin-O-deethylase activity (EROD) (Crespi et al. 1997). EROD activity was increased with co-treatment of BaP and IL-6 compared to BaP treatment alone in both cell lines (Fig. 4a and b). Increase in EROD activity with increasing dose of BaP was seen up to 1 μM, thereafter the EROD decreased (Fig. 4a and b). This decrease in activity was likely due to competitive inhibition of BaP and ethoxyresorufin for CYP1 enzymes (Petrulis and Bunce 1999). In the IL-6 plus PhIP combination experiment, an increase in EROD activity was seen in the MCF-7 cell line (Fig. 4c) but not in the MDA-MB-231 cell line. This pattern of response was similar to that observed for CYP induction in the two cell lines.

**Can IL-6 increase the genotoxicity of dietary carcinogens?**

Since IL-6 treatment resulted in an overall increase in CYP1B1 enzyme expression and CYP1 family enzyme activity (EROD), then it is possible that co-treatment with IL-6 and BaP or PhIP could lead to enhanced genotoxicity of the carcinogens. In initial experiments, we established a dose range of BaP/PhIP that induced a genotoxic response with minimal cell death in mammary cancer cell lines (Table 2). To assess genotoxicity, we used a micronucleus assay.

Both BaP and PhIP were genotoxic in both cell lines, as assessed in the micronucleus assay (Table 2). Over the dose range employed, neither carcinogen was excessively toxic and cytotoxicity levels were within OECD guidelines for the assay. Therefore, to study the effect of co-treatment with IL-6 (500 pg/ml), BaP was used at 100 nM to 10 μM and PhIP was used at 100 nM to 100 μM. The cytotoxicity for all combination experiments did not differ significantly from the negative vehicle control or positive control (etoposide, 1.25 μM) (Fig. 5). In the presence of IL-6 with BaP or PhIP, a significant increase in MN formation was seen compared to BaP or PhIP treatments alone. This IL-6-mediated increase was reproduced in both cell lines. (Fig. 6), however, IL-6 alone did not induce genotoxicity in either cell line (Fig. 6). Etoposide (positive control) is a direct acting genotoxin that does not require metabolic activation and has a completely different mechanism of action (topoisomerase II inhibition) to induce DNA damage, compared to BaP/PhIP. In co-treatments of IL-6 and etoposide, no change in MN formation was observed suggesting IL-6 selectively affects the metabolic activation of pro-carcinogens (Fig. 6).

Since IL-6 does not affect the expression of CYP1A1/2 but does induce CYP1B1 (Figs. 2 and 3), it seems likely that enhanced MN frequency is due to the increased metabolic activity of CYP1B1.

**Can miR27b regulate IL-6?**

Recently, our laboratory has demonstrated that in colorectal cells IL6 can post-transcriptionally regulate CYP1B1 expression through miR27b downregulation via a DNA methylation pathway (Patel et al. 2014). We therefore

### Table 1: Fold change in mRNA expression compared to vehicle control following 24 h treatment with BaP and PhIP in mammary cancer cell lines

| Treatments | MCF-7 | MDA-MB-231 |
|------------|-------|------------|
| BaP 10 nM  | 2.5 ± 0.5 | 2.6 ± 0.3 |
| BaP 100 nM | 8 ± 0.9  | 17.1 ± 1.5 |
| BaP 1 μM  | 24.1 ± 2.2 | 27.1 ± 1.7 |
| BaP 10 μM | 34.2 ± 3.1 | 38.9 ± 4.2 |
| PhIP 100 nM | 1.1 ± 0.6 | 0.5 ± 0.3 |
| PhIP 1 μM  | 1.1 ± 0.6 | 0.3 ± 0.2 |
| PhIP 10 μM | 1.1 ± 0.3 | 0.6 ± 0.4 |
| PhIP 100 μM | 1.7 ± 0.1 | 0.6 ± 0.2 |

Data are fold change compared to the DMSO control presented as a mean ± SEM of at least three independent cultures.

2014; Patel and Gooderham 2015a, b).
explored the possibility that this mechanism may also apply to mammary cells. Treatment of MCF-7 cells with BaP or PhIP alone failed to affect miR27b expression, whereas treatment with IL-6 alone and in combination with BaP and PhIP profoundly reduced miR27b expression (Fig. 7a and c). In contrast in MBA-MD-231 cells, both BaP and IL-6 alone reduced expression of miR27b but PhIP alone did not (Fig. 7b and d). This suggests that IL-6 can regulate the CYP1B1-mediated genotoxicity of BaP and PhIP via miR27b down-regulation in both cell lines.

**BaP induction of IL-6**

This selective effect of BaP (but not PhIP) on miR27b expression in MDA-MB-231 cells (Fig. 7b and d) is potentially of significance. BaP is known to induce inflammation in PBS. Statistically significant differences were calculated using one-way ANOVA with a Dunnett’s post-test (GraphPad Prism 5) (**p < 0.01, *p < 0.05). Data are presented as a mean of at least three independent cultures. Error bars represent the SEM.
in the lung, and Dreij K et al. previously showed that BaP stimulates the production of inflammatory cytokines (IL-6, IL-8) in normal human lung fibroblasts (Dreij et al. 2010), however, we are not aware of any report that links PAHs with the production of cytokines in breast cancer or any mammary cell line. We therefore investigated whether BaP induces IL-6 in mammary cells. In this context, it is noteworthy that the MDA-MB-231 cell line is reported to constitutively express IL-6 (Chiu et al. 1996). We found that there was no change in IL-6 expression in MDA-MB-231 cells treated with BaP for 24 h, but a significant increase in IL-6 expression was seen in a dose-dependent manner following 6 h BaP treatment of cells (Fig. 7e) which is line with the previous reports that IL-6 induction

Fig. 2 Effect of IL-6 on the induction of CYP1 enzymes by BaP and PhIP in MCF-7 cells. CYP1A1 CYP1A2, CYP1B1 induction by BaP (a, b, c) and PhIP (d, e, f) expression were measured by RT-qPCR. Data were normalized to expression of GAPDH and are shown relative to control 0.1% DMSO and 0.1% albumin in PBS (No IL-6). Statistically significant differences compared to no IL-6 and PhIP/BaP alone were calculated using one-way ANOVA with a Dunnett’s post-test (GraphPad Prism 5, ***p < 0.001, **p < 0.01, *p < 0.05). Error bars represent the SEM for independent cultures (n = 3)
showed rapid kinetics (Gan et al. 1991). We therefore examined whether BaP-driven miR27b and IL-6 mRNA expression were associated and found a negative correlation ($p < 0.0001$, Pearson’s product moment correlation), suggesting BaP alone can enhance the inflammatory environment by up-regulating IL-6 gene expression in cells constitutively expressing this gene (MDA-MB-231), leading to reduction in miR27b expression.

Fig. 3 Effect of IL-6 (500 pg/ml) on the induction of CYP1 enzymes by BaP and PhIP in MDA-MB-231 cells. CYP1A1, CYP1A2, CYP1B1 induction by BaP (a, b, c) and PhIP (d, e, f) expression were measured by RT-qPCR. Data were normalized to expression of GAPDH and are shown relative to control 0.1% DMSO and 0.1% albumin in PBS (No IL-6). Statistically significant differences between IL-6 and BaP/PhIP alone were calculated using one-way ANOVA with a Dunnett’s post-test (GraphPad Prism 5, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). Error bars represent the SEM for independent cultures ($n = 3$).
miR27b and CYP1B1 expression

To further confirm if miR27b can regulate CYP1B1 expression, cells were transfected with miR27b inhibitor. Both MCF-7 and MDA-MB-231 cells express endogenous low levels of miR27b, we therefore used a chemically modified antisense oligonucleotide miRNA inhibitor designed to bind and sequester the complimentary mature microRNA strand (Stenvang et al. 2012). MCF-7 and MDA-MB-231 cells were transfected with scrambled oligonucleotide or miR27b inhibitor using LIPO2000 for up to 72 h (Dalby et al. 2004). Optimal inhibition of miRNA expression was determined to be 24 h for MCF-7 cells and 48 h for MDA-MB-231 cells.

Measurement of miRNA levels indicated miR27b was decreased significantly in both cell lines transfected with miR27b inhibitor (Fig. 8a and c). Importantly, significant reduction of miR27b expression was accompanied with a significant increase in CYP1B1 expression (Fig. 8b and d), supporting the proposal that miR27b is involved in the regulation of CYP1B1 in these mammary cell lines.
Table 2  Cytotoxicity and micronucleus frequency following 24 h treatment with different doses of BaP and PhIP in mammary cancer cell lines

| Treatments | MCF-7 Cytotoxicity<sup>a</sup> | Micronuclei<sup>b</sup> | MDA-MB-231 Cytotoxicity<sup>a</sup> | Micronuclei<sup>b</sup> |
|------------|-------------------------------|-------------------------|-----------------------------------|-------------------------|
| Control    | 90.0 ± 2.7                    | 3.2 ± 0.8               | 88.3 ± 1.2                        | 7 ± 0.9                 |
| BaP 10 nM  | 90.1 ± 2.3                    | 9.7 ± 2.5               | 87.3 ± 1.7                        | 37.8 ± 10               |
| BaP 100 nM | 87.2 ± 3.9                    | 16 ± 1.9                | 87.3 ± 2.3                        | 40.3 ± 12.3             |
| BaP 1 µM   | 85.9 ± 2.3                    | 29.7 ± 4.5              | 86.1 ± 4.9                        | 65.2 ± 9.6              |
| BaP 10 µM  | 83.5 ± 1.0                    | 49.8 ± 11.9             | 82.4 ± 0.7                        | 114.7 ± 8.9             |
| Etoposide  | 81.7 ± 1.4                    | 124.3 ± 28.7            | 83.6 ± 2.6                        | 321.7 ± 51.1            |
| Control    | 89.0 ± 1.0                    | 3.5 ± 0.5               | 89 ± 7.2                          | 3.5 ± 1.0               |
| PhIP100 nM | 86.0 ± 2.4                    | 12.8 ± 5.1              | 87.2 ± 1.5                        | 3.2 ± 2.5               |
| PhIP1 µM   | 85.6 ± 2.4                    | 22.3 ± 2.6              | 88.1 ± 0.9                        | 4.7 ± 1.8               |
| PhIP10 µM  | 85.8 ± 2.6                    | 29.2 ± 3.4              | 90.4 ± 4.3                        | 9.0 ± 0.9               |
| PhIP100 µM | 82.7 ± 1.7                    | 49 ± 3.5                | 87.7 ± 1.5                        | 13.7 ± 1.3              |
| Etoposide  | 81.7 ± 1.4                    | 172 ± 4.8               | 83.7 ± 2.2                        | 121.0 ± 27.7            |

Values are mean ± SEM, for three independent cultures
<sup>a</sup>Cytotoxicity is expressed as % viable cells
<sup>b</sup>Micronuclei frequency per 1000 cells

Fig. 5  Cytotoxicity of carcinogens in the presence of IL-6 (500 pg/ml) in breast cells, in MCF-7 (a, b) and MDA-MB-231 (c, d) cells treated with BaP or PhIP in the presence or absence of IL-6 for 24 h. Cells were harvested 48 h post-treatment. Cytotoxicity was measured by cell counting using haemocytometer and is expressed as % of cell survival. No significant change in cell survival was observed.
In breast cancer, elevated circulating levels of pro-inflammatory cytokine IL-6 have been linked with poor prognosis (Zhang and Adachi 1999; Fouad et al. 2014), and consumption of a pro-inflammatory diet is also associated with an increased incidence of breast cancer (Shivappa et al. 2015). These results suggest that IL-6 can drive carcinogenesis leading to breast cancer and dietary induction of IL-6 can contribute to this, but the underlying mechanism remains unclear. Here using established mammary chemical carcinogens, we propose that IL-6 can enhance the activation of dietary carcinogens leading to genetic damage in mammary cells as summarized in Fig. 9.

The current study presents mechanistic evidence that at levels reported to be circulating in clinical cancer (Blay et al. 1992; Bachelot et al. 2003; Goswami et al. 2013; Brouwers et al. 2015) and present in breast tumors (Ueno et al. 2000; Knupfer et al. 2004; Knupfer and Preiss 2007), IL-6 affects the metabolic competency of mammary cells leading to an increase in the genotoxicity of CYP-activated chemical carcinogens such as BaP and PhIP. Both compounds are pro-carcinogens present in smoke and cooked food that require metabolic activation to exert their genotoxicity (Winton et al. 1990; Brooks et al. 1994, 1999; Gooderham et al. 1996, 1997, 2002; Yadollahi-Farsani et al. 1996). To the best of our knowledge, this is the first report to show the effect of interleukins on the genotoxicity of dietary carcinogens in mammary cell lines, although, TNF-α (pro-inflammatory cytokine) was shown to increase the genotoxicity of BaP in alveolar epithelial type II cells (Umannova et al. 2008) and IL-8 has been shown to increase BaP metabolism in human lung epithelial cells (Shi et al. 2017). Recently, our laboratory has reported that IL-6 can increase the genotoxicity of...
BaP/PhIP by regulation of CYP1B1 expression in colorectal cells (Patel and Gooderham 2015a, b).

Extending this finding to mammary cells, we here show that IL-6 can induce CYP1B1 via a miR27b mechanism leading to increased bio-activation of BaP/PhIP and that this has genotoxic consequences in two different mammary cell lines. IL-6 treatment failed to change the level of CYP1A1 or CYP1A2 expression and activity, but it did increase CYP1B1 gene expression. Previously it was generally accepted that under conditions of inflammation or infection, hepatic CYP enzyme activity tended to be depressed (Deloria et al. 1985; Gooderham and Mannering 1986; Morgan 1989; Renton 2004; Aitken et al. 2006). Consistent with this, IL-6 represses the inducible expression of CYP1As.

Fig. 7 Expression of miR27b by BaP and PhIP in presence or absence of IL-6 in MCF-7 (a, c) and MDA-MB-231 cells (b, d): miRNA27b was measured by RT-qPCR. Data were normalized to expression of U6 and is shown relative to control 0.1% DMSO and 0.1% albumin (No IL-6). e: IL-6 gene expression after 6 h BaP treatment. Significant differences were calculated using one-way ANOVA with a Dunnett’s post-test (GraphPad Prism 5, ***p < 0.001, **p < 0.01, *p < 0.05) and are shown for comparisons between control vs treated samples. Error bars represent SEM for independent cultures (n = 3)
and CYP3A4 in human hepatocytes (Muntane-Relat et al. 1995) and IL-6 plasma levels are inversely related to hepatic CYP1A2 and CYP2E1 expression in cardiac arrest patients (Frye et al. 2002). Down-regulation of CYP1A2 and CYP2E1 is IL-6 mediated in mouse hepatocytes (Siewert et al. 2000) and IL-1β, IL-6, tumor necrosis factor-alpha and interferon-gamma reduce CYP1A1, 1A2 and 3A6 expression in rabbit hepatocytes (Bleau et al. 2003). Importantly, each of these studies has examined hepatic drug metabolism activity, whereas the current study used mammary cells.

Fig. 8 MiR27b and CYP1B1 gene expression after transfection with miR27b inhibitor; miR27b expression in MCF-7 (a) and MDA-MB-231 (c), CYP1B1 mRNA expression in MCF-7 (b) and MDA-MB-231 (d) after transfection with miR27b inhibitor (25 µM) for 24 h in MCF-7 cells and 48 h in MDA-MB-231 cells. Statistically significant differences were calculated using Student’s t test (GraphPad Prism 5) control scramble oligo vs treated miR27b inhibitor. (***p < 0.001, **p < 0.01, *p < 0.05). Error bars represent SEM for independent cultures (n = 3)
These observations along with the current work and that previously reported (Patel et al. 2014; Patel and Gooderham 2015a, b) suggest that systemic inflammation elicits tissue-specific effects on xenobiotic metabolism. The selective induction of CYP1B1 metabolic competency in mammary cells exposed to clinically relevant concentrations of IL-6 has potentially important clinical significance. Our data show that under such conditions, mammary cells exposed to IL-6 acquire increased metabolic competency to activate dietary/environmental carcinogens such as BaP/PhIP promoting genotoxic/mutagenic events. In this context, a number of clinical studies in different populations have linked high CYP1B1 expression with an increase in the risk of breast cancer (Gaudet et al. 2006; Jiao et al. 2010; Blackburn et al. 2015). In addition to a role in xenobiotic carcinogen activation, it is known that CYP1B1 catalyzes the conversion of 17-β-estradiol to catechol estrogen metabolites that promote the invasiveness of ER responsive breast cancer (Gaudet et al. 2006; Jiao et al. 2010; Blackburn et al. 2015). Furthermore, BaP and PhIP are present in cigarette smoke (Manabe et al. 1991; Pfeifer et al. 2002) and active smoking is linked with increased incidence of ER positive mammary cancers (Kawai et al. 2014). This is consistent with results from this study and previous studies from our laboratory (Lauber et al. 2004; Lauber and Gooderham 2007, 2011; Patel et al. 2014; Malik et al. 2018a, b), which report the estrogenicity of BaP and PhIP in addition to their well-documented metabolically mediated DNA-damaging properties.

This study and previous reports propose that IL-6 can influence miR7b expression to regulate the expression of CYP1B1, which coincidentally is highly expressed in breast cancer (Gaudet et al. 2006; Jiao et al. 2010; Blackburn et al. 2015). Overexpression of CYP1B1 is not only linked with increased risk of breast cancer but also shown to decrease the sensitivity of certain anti-cancer drugs (e.g., docetaxel, flutamide) leading to poor prognosis (Rodriguez-Antona and Ingelman-Sundberg 2006). Therefore, CYP1B1 is considered an attractive therapeutic target in breast cancer (McFadyen et al. 2004; Safe et al. 2013). In this study, we have confirmed that miR27b has the potential to selectively target CYP1B1 expression in mammary cells, and thus manipulation of miR27b activity could potentially play a role in attenuating key DNA damage in cancer.

Another important finding of the present study was elevation of IL-6 following BaP treatment of the mammary epithelial cells, suggesting that this dietary carcinogen is itself capable of inducing inflammation. This we reported in a previous paper that described the potential of BaP to upregulate inflammation within the microenvironment to influence cell migration and invasion (Malik et al. 2018a, b). Consistent with this, others have shown that BaP stimulates the production of inflammatory cytokines (IL-6, IL-8) in normal human lung fibroblasts (Dreij et al. 2010). Thus, we have established a cyclical mechanism where exposure to a human carcinogen promotes inflammation that in turn can induce metabolic activation of the carcinogen leading to genotoxic consequences (Fig. 9) and thereby carcinogenesis.

The consumption of meat has been associated with the development of cancer including breast cancer. Interestingly, a prospective study showed that consumption of red meat can result in elevated levels of inflammatory biomarkers in women (Ley et al. 2014) and pro-inflammatory diet intake can increase the incidence of breast cancer (Shivappa et al. 2015). Epidemiological studies suggest that risk of cancer in people consuming red meat, particularly processed meat,
is higher compared to non-eaters (Sinha and Rothman 1999; Wu et al. 2009).

These epidemiological studies all support an association between meat intake, inflammation and cancer. Here we have reported a mechanistic explanation for these trilateral associations showing the influence of inflammation on the metabolic activation of food-derived genotoxins by CYPs and the potential genotoxic consequences of these events.

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**Compliance with ethical standards**

**Conflict of interest** All authors declare that they have no actual or potential conflicts of interest.

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