



## Curcumin Inhibit PhIP-Induced Carcinogenicity by Regulating Expression of Nrf2 and FOXO Targets, and BRCA-1 and P16 Expression in Breast Epithelial Cells

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### Brief Report

PhIP (Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) is a heterocyclic amine (HCA) which is formed when meat products are cooked at high temperature. PhIP is known for its genotoxic and carcinogenic effects causing several types of cancer, including breast cancer. HCA causes multifold cytotoxic effect, for example metabolism of PhIP leads to ROS production, and PhIP metabolites produce DNA adduct and DNA strand breaks [1-4]. Breast epithelial cells contain all the machinery to metabolize HCA and the genotoxic effects of these metabolites may lead to breast cancer [1].

The prevention of cancer through diet is categorized as one of the most effective ways to reduce cancer incidence [5]. We hypothesized that curcumin may be a potential food additive that may be inhibitory to PhIP-induced carcinogenicity by inhibiting ROS production, DNA adduct formation and DNA strand breaks. We developed a model system using the breast epithelial cells (MCF-10A) to screen several dietary additives to identify phytochemical that is capable to inhibit PhIP cytotoxicity. Curcumin a polyphenol and major component of the Indian spice turmeric, which is used in various food preparations, is known to inhibit cell proliferation and has anticancer effects [6]. In this brief report, we describe how curcumin inhibits PhIP-induced ROS production, DNA adduct formation and DNA damage in MCF-10A cells.

MCF-10A human breast epithelial cells were cultured in a humidified incubator at 37°C under 5% CO<sub>2</sub> atmospheric conditions in RPMI media supplemented with 10 µg/ml insulin, 20 ng/ml epidermal growth factor, 10 mg/ml hydrocortisone, 5% horse serum and 1% penicillin-streptomycin (10,000 U/ml). Cells were treated with or without PhIP (50 and 250 µM) in the presence or absence of curcumin (150 µM) and cell viability determined using the cell counting kit-8 (Dojindo Laboratories). Cells were pretreated with curcumin 15 minutes prior to dosing with PhIP (50 or 250 µM). MCF 10A cells viability is decreased when treated with PhIP in a dose dependent manner. Results shows that curcumin at a concentration of 150 µM significantly inhibited PhIP-induced reductions in viability at 24 h, with cells treated with 50 µM PhIP plus 150 µM curcumin, and 250 µM PhIP plus 150 µM curcumin (Table 1a).

The antioxidant capacity of curcumin was analyzed in the absence and in the presence of the PhIP, a well-known peroxidant agent. Its efficiency was evaluated in terms of inhibition of intracellular reactive oxygen species (ROS) production induced spontaneously or in the presence of PhIP. Intracellular free radical species were detected by measuring the fluorescence intensity values (using a Biotek, Synergy HT instrument with an excitation 475-495 and emission 518-528) due

to the oxidation of DCF and expressed as relative fluorescence units (RFU).

In the absence of PhIP, ROS production was limited but increased significantly in the presence of PhIP in a dose-dependent manner. Co-treatment of MCF-10A cells with curcumin resulted in a significant decrease in PhIP-induced ROS production (Table 1b).

(a) Cell Viability (%)	Mean
Control	100
PhIP 50 mM	80
PhIP 50 mM+Cur 25 mM	78
PhIP 50 mM+Cur 50 mM	81
PhIP 50 mM+Cur 75 mM	92*
PhIP 50 mM+Cur 100 mM	97***
PhIP 50 mM+Cur 150 mM	101***
PhIP 50 mM+Cur 200 mM	81
PhIP 250 mM	33
PhIP 250 mM+Cur 25 mM	49*
PhIP 250 mM+Cur 50 mM	69***
PhIP 250 mM+Cur 75 mM	74***
PhIP 250 mM+Cur 100 mM	86***
PhIP 250 mM+Cur 150 mM	97***
PhIP 250 mM+Cur 200 mM	66***
(b) ROS Activation (Mean Relative Fluorescence Units)	
Control	3.33
PhIP 50 mM	6.33***
PhIP 250 mM	8.33***
PhIP 50 mM+Cur 150 mM	3.33***
PhIP 250 mM+Cur 150 mM	2.66***
Cur 150 mM	2
H2O2 1 mM	5
H2O2 10 mM	10.6

(c) Comet Assay (Mean Olive Tail Moment)	
Control	0.155
PhIP 50 $\mu$ M	1.02***
PhIP 250 $\mu$ M	1.5***
PhIP 50 $\mu$ M+Cu 150 $\mu$ M	0.26***
PhIP250 $\mu$ M+Cu 150 $\mu$ M	0.53***
Cu 150 $\mu$ M	0.17

(a) Cell viability with various doses of curcumin co-treated with 50  $\mu$ M and 250  $\mu$ M PhIP. All doses of curcumin produced reversal of PhIP-induced cytotoxicity but curcumin at 150  $\mu$ M exhibited the greatest degree of reversal of cytotoxicity. Line shows significance compared to PhIP treated samples.

(b) Quantitative fluorescence intensity analysis of ROS production in PhIP-treated MCF-10A cells upon co-treatment with curcumin. MCF-10A cells were treated with or without PhIP in the presence and absence of curcumin for 24 hours and then fluorescence intensity was measured with DCF-DA (5  $\mu$ g) within 45 minutes. Co-treatment with curcumin reduced the PhIP-stimulated ROS production in MCF-10A cells.

(c) Inhibition of PhIP-induced DNA strand breaks by curcumin at 24h. MCF 10A cells were treated and analyzed to score comet tail moment using comet analysis system (Loats Associate System, Westminster, MD). Values are mean from three independent experiments performed in triplicate. The statistical significance is expressed as \* $p$ <0.05, \*\* $p$ <0.01, and \*\*\* $p$ <0.001.

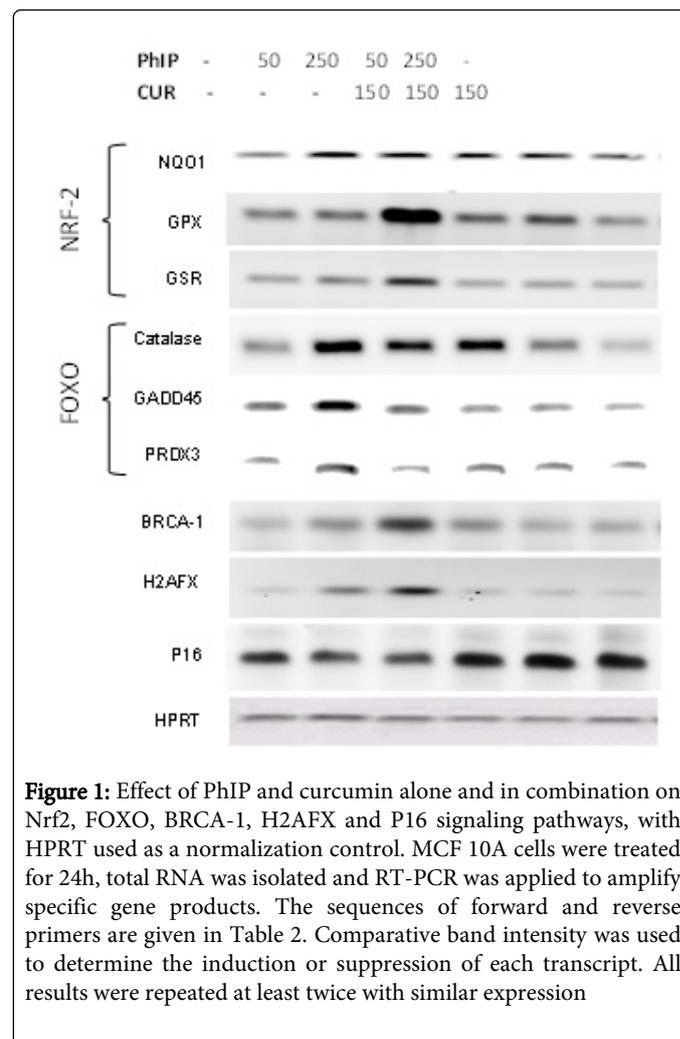
**Table 1:** Effect of curcumin on inhibition of PhIP induced carcinogenicity.

DNA adduct formation was determined using an immunofluorescence method with an anti-DNA adduct primary antibody [8]. DNA adducts accumulated in a dose-dependent manner in both 50 and 250  $\mu$ M PhIP-treated breast epithelial MCF 10A cells. When MCF 10A cells were co-treated with curcumin, PhIP-induced DNA adduct formation was noticeably reduced [7].

The ability of curcumin to inhibit PhIP-induced DNA double strand breaks in MCF-10A cells was then determined using the comet assay and measuring the olive tail moment. Treatment with curcumin (150  $\mu$ M) had no effect on the production of DNA strand breaks compared to the controls. However, pretreatment with curcumin inhibited DNA double strand breaks induced by PhIP after 24 h (Table 1c). These results also support the DNA adduct data since the reduction in DNA adduct formation in the presence of curcumin represents decreased DNA damage.

To understand the interaction of PhIP and curcumin at the molecular level reverse transcriptase PCR (RT-PCR) was performed. Oxidative stress signals through NRF-2 [Nuclear factor (erythroid-derived 2)-like 2] and its targets such as NQO-1 [NAD(P)H quionine oxidoreductase-1], GPX-1 [glutathione peroxidase] and GSR [glutathione reductase], as well as FOXO [forkhead box protein] targets such as CAT [catalase], GADD-45 [growth arrest and DNA damage-inducible 45] and PRDX-3 [Thioredoxin-dependent peroxide reductase], and the expression of these genes was monitored. H2AX [histone H2A], BRCA-1 [breast cancer 1, early onset] and P-16 (cyclin-dependent kinase inhibitor 2A) were also evaluated. PhIP induces the expression of NQO1, GPX-1, GSR, Catalase, GADD45, PRDX-3, BRCA-1 and H2AFX. However, curcumin inhibited the PhIP-elevated expression of these genes. Whereas the expression of cyclin-dependent kinase inhibitor 2A (P16), which is also known as multiple tumor suppressor protein, is suppressed by PhIP as compared

to control; however, P16 expression was maintained in breast epithelial cells co-treated with curcumin. Since MCF-10A cells are P53 deficient, the expression of the P16 tumor suppressor is important to reduce PhIP carcinogenicity. Thus, down-regulation of the P16 transcript by PhIP could result in a carcinogenic effect, such that maintenance of P16 expression by curcumin suggests that this agent should reduce PhIP-induced carcinogenicity. The house-keeping gene hypoxanthine phosphoribosyl transferase (HPRT) was expressed uniformly in all groups (Figure 1).



**Figure 1:** Effect of PhIP and curcumin alone and in combination on Nrf2, FOXO, BRCA-1, H2AFX and P16 signaling pathways, with HPRT used as a normalization control. MCF 10A cells were treated for 24h, total RNA was isolated and RT-PCR was applied to amplify specific gene products. The sequences of forward and reverse primers are given in Table 2. Comparative band intensity was used to determine the induction or suppression of each transcript. All results were repeated at least twice with similar expression

Gene	Forward Primer	Reverse Primer
NQ1	5'-TTA CTA TGG GAT GGG GTC CA	5'-AAA MC CAC CAG TGC CAG TC
GPX	5'-CTT ACA GTG CTT GTG CGG GG	5'-GTT CTT GGC GTT CTC CTG AT
GSR	5'-GCA TCT ATG CAG TTG GGG AT	5'-GTA GGG TGA ATG GCG ACT GT
Catalase	5'-CGT GCT GAA TGA GGA ACA GA	5'-TCT TCA TCC AGT GAT GAG CG
GADD45	5'-AAA GGA TGG ATA AGG TGG GG	5'-TCC CGG CM MA CM ATA AG

PRDX3	5'-TCT GGT TCC AGT CAA GCA AA	5'-CGT AGT CTC GGG AM TCT GC
BRCA1	5'-TTGCGGGAGGAAAATGGGTAG TTA	5'-TGTGCCAAGGGTGAATGATGA AAG
H2AFX	5'-TTGCCCGCAGTCTGAAGCG	5'-CCGGCMGAAGGCCACCCAGG
P16	5'-GTGTGGCACATCCCGCCCTC	5'-CGCAAGCTGGCTGGCTCACT
HPRT	5'-GCGTCGTGATTAGCGATGATG AAC	5'-CCTCCCCTCTCCTTCATGACAT CT

**Table 2:** Primers sequence used in RT PCR.

Breast cancer is one of the leading causes of death in women [9,10], and diet plays a major role in development of the disease [5]. A direct relationship between red meat consumption and the PhIP associated development of breast cancer has been demonstrated [11]. This is the first study demonstrating the mechanism by which curcumin can inhibit the carcinogenic effect of PhIP in MCF-10A breast epithelial cells. Normal breast epithelial cells have an inherent capacity to bioactivate PhIP which then causes DNA damage. Our cytotoxicity data reveals that curcumin can inhibit PhIP-induced cell death. Bio-activated PhIP also causes the production of ROS; the effect of PhIP on both these processes has been established previously [12]. PhIP-induced DNA adducts and ROS lead to DNA double strand breaks [7]. The cumulative effect of these factors affects cells' normal behavior and is responsible for the decrease in cell viability. Many studies demonstrate that an imbalance in the production and detoxification of ROS may lead to various cancers [13,14]. Our results show that curcumin reduced DNA adduct formation, decreased DNA double strand breaks and reduced ROS production to basal levels to result in an inhibition of PhIP-induced cell death [7]. Although PhIP induces antioxidant and DNA repair mechanisms through the Nrf2 and FOXO pathways, this response does not completely inhibit ROS or DNA adduct formation. Curcumin, however, inhibits both ROS and DNA adduct generation to rescue DNA damage. We conclude that in addition to DNA adduct formation, oxidative DNA damage is crucial to PhIP-induced carcinogenicity. Previously, Sato et al., [15] have shown that ROS production during the metabolism of heterocyclic amines including PhIP occurs through NADPH/P450, suggesting that production of ROS in PhIP-treated MCF-10A cells is due to the metabolism of PhIP to N-hydroxy-PhIP through p450 detoxification. Therefore, DNA double strand breaks in PhIP-treated cells are likely to arise through two mechanisms: (i) the ROS generated directly contribute to DSB; and (ii) PhIP-DNA adduct formation leads to DNA strand breaks [7].

Our results clearly show that many antioxidant genes are induced in the presence of PhIP. Both the Nrf2 and FOXO pathways are up-regulated by PhIP to scavenge the elevated ROS and protect cells from DNA adduct formation and the resulting DNA damage. Increased expression of H2AFX and BRCA-1 in the PhIP-treated group as well as data from comet assays, ROS monitoring and immunofluorescence with anti-DNA adduct antibodies support this idea [7]. Previous studies have shown that BRCA-1, P-53 and other tumor suppressor genes are able to increase GADD-45 expression [16-18]. Catalase and GADD-45 are associated with the FOXO pathway; however, these two genes perform different functions of detoxification and DNA repair,

respectively. In our study, PhIP treatment increased the expression of BRCA-1 indicating that oxidative stress was induced by PhIP (also confirmed by the DCF assay); curcumin co-treatment reduced the oxidative stress allowing the expression of BRCA-1 to return towards basal levels. Studies have shown that BRCA-1 regulates Nrf2-dependent antioxidant signaling by physically interacting with Nrf2 and promoting its stability and activation [19]. Since curcumin inhibits both PhIP-induced ROS production and DNA adduct formation, this agent ultimately reduces DNA-DSB. In addition, curcumin might improve DNA repair mechanisms, and together these responses reduce the possibility of DNA mutations. Such multiple mechanisms of action of curcumin in cancer cells has been documented previously [20]. Our results also indicate that curcumin modulates PhIP-induced effects through the regulation of multiple cell signaling pathways including antioxidant, DNA repair, tumor suppressor pathways (p16) to minimize the damage caused by the food carcinogen PhIP [7].

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