EFFECTS OF CURCUMIN ON POLYCHLORINATED BIPHENYLS EXPOSED F98 GLIOMA CELLS

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Summary


Curcumin (commonly called turmeric) was shown to have potential in the prevention and treatment of malignancies. Among these, glioblastomas are the most common lethal and least chemotherapeutically responsive primary malignancy of the central nervous system (CNS). Since curcumin has neuroprotective effects especially in terms of oxidative stress mechanisms which is confirmed in in vivo studies; the effects of curcumin on Aroclor 1254 treated F98 rat glioblastoma cells were investigated in vitro to evaluate the effects in cellular level of co-preincubation. Preincubation with curcumin at 3 µg/mL were found to inhibit the proliferative effects of Aroclor 1254 at 10 ng/mL where the cytotoxicity values were increased to 76.44%. Preincubation with curcumin was shown to be more efficient compared to coinbubation. In terms of duration, coinubation for 24 h was found to be more efficient in the inhibition compared to 4-hour treatment. The current study supports the cytotoxic effects of curcumin on glioma cells along with its antiproliferative effects on cell proliferation induced by the environmental carcinogen polychlorinated biphenyls (PCBs).

Key words: Aroclor 1254 toxicity, curcumin, glioma cells, in vitro assays

INTRODUCTION

Glioblastoma multiforme, the most common lethal primary malignancy (with grade IV) of the central nervous system (CNS) is among the least therapeutically responsive tumours (Wilson et al., 2014). Several in vitro models were developed for simultaneous evaluation of potential therapeutics and possible synergies with alternatives to increase the survival and improve the quality of life of the patients.
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with glioblastoma (Gentile et al., 2015; John et al., 2017).

Curcumin (diferuloylmethane) as a traditional spice and ethnomedicinal agent, is the principal curcuminoid of turmeric derived from the plant Curcuma longa Linn. and approved by United States Food and Drug Administration as "Generally Recognized as Safe" where it could be used for the medical treatment without review by authorities. This traditional compound was shown to have antioxidant, anti-infective and anticancer effects confirming its wide use in several chronic diseases including diabetes, cardiovascular diseases, gastrointestinal-neurodegenerative disorders and some psychiatric conditions (Gupta et al., 2013).

For therapeutic effects on neoplastic changes, curcumin was found promising for pancreatic, hepatocellular, gastric, breast, prostate, skin, lung and colon cancers, myeloma and glioblastomas (Fadus et al., 2017). Antitumoural effects of curcumin were attributed to the activation of apoptotic pathways, induction of autophagy, disruption of molecular signalling pathways, decrease of invasion and metastasis through various molecular mechanisms (Fadus et al., 2017). Curcumin was suggested to be complementary as a second-line therapy after failure of radiation therapy and temozolomide with combination of the cytotoxic compounds carmustine or lomustine in glioblastoma multiforme patients (Sordillo et al., 2015).

Due to their persistence and lipophilicity, polychlorinated biphenyls (PCBs) tend to biocumulate in cerebrospinal and bioaccumulate causing endocrine disruption, immunosuppression, neurotoxicity and carcinogenesis, leading to emerging public and environmental risk (Robertson & Ludewig, 2011). Strong evidence is linked to liver and biliary tumours induced by PCBs designating them as Group 1 by the International Agency for Research on Cancer, while suspected evidence is related to breast and non Hodgkin lymphoma (NHL) for this compound (Clapp et al., 2008). Epidemiological studies raised concern on the relation of this environmental pollutant, and brain tumours including glioblastoma multiforme (Petruska & Engelhard, 1991). Exposure to the PCB mixture Aroclor1254 was found to alter glial cell differentiation through induction of perturbation in cyclic AMP/protein kinase A (cAMP/PKA) and protein kinase C (PKC) signalling pathway (Adornetto et al., 2013). Curcumin was found to inhibit effectively amyloid beta-peptide accumulation (Abeta) and beta-amyloid oligomers and fibrils formed by Abeta as confirmed in both in vivo and in vitro studies; which is important in Alzheimer’s disease pathology (Ono et al., 2004; Lee et al., 2013). The overexpression of aggregated alpha-synuclein (AlfaS) related to Parkinson disease, as the second most common neurodegenerative disease, was found to be inhibited by curcumin through the inhibition of cytotoxicity due to the increased reactive oxygen species (ROS), oxidative stress and mitochondrial disfunctions in an in vitro model using SH-SY5Y-human neuroblastoma cells (Wang et al., 2012). As some neurotoxins and pesticides increase nonfibrillar oligomer and protofibrils of AlfaS through increased ROS production causing apoptosis, curcumin was suggested as a promising compound to inhibit this process (Lee et al., 2013).

Previously, we have shown the neuroprotective effects of curcumin on CNS damage in offspring of pregnant rats (7 to 21 gestation days) exposed to Aroclor 1254 (PCB mixture) at 1 mg/kg body weight. According to our unpublished
data, Aroclor 1254 alone induced neurodegeneration; while in combination with curcumin the lesions disappeared or degraded compared to control group supported by oxidative stress parameters (Alcigir et al., 2016). Protective effect of curcumin were evaluated in two different methods in vitro; compromising the prophylactic (curcumin coincubation group) and the preventive (curcumin preincubation group) effects. In order to exert its antioxidant effect; curcumin needs to reach a steady state condition at the treated cells; therefore, the antiproliferative effect is expected to be higher in pre-treatment group compared to coincubation. Therefore, provided that PCBs increase ROS activation which might eventually increase nonfibrillar oligomer and protofibrils of AlfaS; curcumin inhibition of this process along with its antiproliferative properties and the in vivo preliminary findings supporting this protective activity, the aim of this study was to confirm in vitro curcumin’s efficacy on PCB treated rat glioma cell line F98 in pre- and coincubation routes.

MATERIALS AND METHODS

Cell culture

F98 rat glioma cell lines (ATCC® CRL-2397TM) were cultured according to Gil et al. (2011). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% PBS (phosphate buffer saline solution) (Gibco/BRL, Frederick, MD) and 1% antibiotic-antimycotic solution (Invitrogen, 15240-112) at 37 °C in a humidified carbondioxide incubator at 5% (Nuve EC160, Turkey). Prior to treatment, the cells were expanded to ~85% confluency in 96-well plates which were automatically calculated by Juli FL Live Cell Analyzer (NanoEnTek Inc, Seoul, South Korea). The dose selection for this initial experiment was adjusted from the studies by Madia et al. (2004); Gao et al. (2005); Kratzer (2012). Cells were primarily treated with the Aroclor 1254 and Curcumin at 0.082–166.67 µg/mL concentrations alone (12 doses in half dilutions) for the determination of half maximal inhibitory concentration (IC50). Dose selection for the coincubation groups was done according to the calculated IC50 values. For curcumin, since these values were ~8 and ~10 µg/mL by MTT and Neutral Red assays; the highest concentration was selected as 6 µg/mL and applied at five concentrations in half dilutions corresponding the lowest dose at 0.375 µg/mL. For Aroclor1254, IC50 values ranged ~40 and 42 ng/mL; so the selection of the co-incubation initiated at the highest concentration to be applied as 40 ng/mL and applied in four concentrations at half dilutions with the lowest at 5 ng/mL. For pre-incubation, two concentrations from each drug were tested with the concentrations of 10 and 20 ng/mL Aroclor 1254 and 1.5 and 3 µg/mL for curcumin.

Coincubation groups, where both drugs were applied simultaneously were both tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT assay) at post exposure hours 4 and 24. For preincubation groups, cells were treated with curcumin for 24 hours, where Aroclor was later added to the fresh medium and evaluated in terms of MTT activity at the following 24 hours. All concentrations were applied in triplicate where the experiment is repeated with three different passages from the F98 making a dataset of nine (n=9) for each dose.
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Cytotoxicity assays

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (conventionally known as MTT) assay and Neutral Red (NR) assay were performed according to Mossman (1983) and Repetto et al. (2008), and the optic density was read at 540 nm using a microplate reader (SpectraMax®i3 multi-mode microplate reader; Molecular Device, Sunnyvale, CA, USA). Cytotoxicity was calculated with regard to the untreated cell control, which was set to 100% viability (maximal viability, MaxV). The dead cell control (Triton-X) was set to 0% viability (minimal viability, MinV). The degree of cytotoxicity of drug-treated cells is expressed as a percentage of the untreated cell control. A plot of % cytotoxicity versus sample concentrations was used to calculate the concentration which showed 50% cytotoxicity (IC\textsubscript{50}).

Anti-proliferative effect

Antiproliferative effects on treated cells were calculated using the standard trypan blue technique through JuLI\textsuperscript{TM} Br Counting starter kit (NanoEnTek Inc, Seoul, South Korea). A 10 μL sample was mixed with 10 μL of 0.4% trypan blue stain. Ten μL from this well-mixed sample were loaded on JuLI\textsuperscript{TM} Br Cell counting slide where the live-dead and total cells were calculated automatically.

Statistical analysis

Measured data were plotted against the corresponding inhibition values, resulting in the inhibition curves as regression analysis, selected by the highest correlation coefficient (R’) using NCSS (2007). IC\textsubscript{50} (half maximal inhibitory concentration) values were calculated by interpolation of experimental data by prediction outcomes using Graph Pad® Prism 4.0. The differences between the dose and percent cytotoxicity values were evaluated by Mann Whitney U and Chi square tests according to the homogeneity of test results. A minimum of 5% significance level was considered for all comparisons (P>0.05).

RESULTS

Cytotoxicity

IC\textsubscript{50} values for curcumin and Aroclor 1254 by MTT and NR assays are given in Table 1. Since no dose response relationship was observed by LDH assay, IC\textsubscript{50} values could not be determined. According to IC\textsubscript{50} values by MTT and NR assay, coinubcation concentrations were selected. Morphological changes are shown in Fig. 1.

The results of the coinubcation for 4 h with curcumin (0, 0.375, 0.75, 1.5, 3 and 6 μg/mL) and Aroclor (5, 10, 20, 40 ng/mL) (Table 2, Fig. 2); indicate that eventhough no dose-response was observed, highest cytotoxic activity was present at 40 ng/mL Aroclor incubation with 6 μg/mL curcumin (85.62 %); while this was insignificant (p>0.05) compared to other treatment concentrations. Both drugs at lowest concentration resulted in cell proliferation.

| Table 1. IC\textsubscript{50} values of Aroclor 1254 and curcumin on F98 cells |
|-----------------|----------|----------|-----------------|
|                  | MTT Assay | LDH Assay | Neutral Red Assay |
| Aroclor 1254 (ng/mL) | 39.540    | ND*       | 42.440           |
| Curcumin (μg/mL)    | 8.136     | ND*       | 10.364           |

* ND=Not detected.
Similar to 4 h incubation, Aroclor 40 ng/mL and curcumin at 6 µg/mL induced the highest cytotoxic effect (70.16 %) after 24 h incubation. The dose-response for curcumin were more evident at 40 ng/mL Aroclor treated cells compared to other concentrations (Table 3, Fig. 3).

Table 2. Percent cytotoxicity of curcumin and Aroclor 1254 after coincubation for 4 hours (mean±SD; n=9)

<table>
<thead>
<tr>
<th>Curcumin (µg/mL)</th>
<th>Aroclor 1254 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>85.62±30.15</td>
</tr>
<tr>
<td>3</td>
<td>82.47±30.60</td>
</tr>
<tr>
<td>1.5</td>
<td>72.92±24.58</td>
</tr>
<tr>
<td>0.75</td>
<td>50.28±24.05</td>
</tr>
<tr>
<td>0.375</td>
<td>46.37±19.71</td>
</tr>
<tr>
<td>Aroclor 1254 alone</td>
<td>73.89±13.63</td>
</tr>
</tbody>
</table>

For 24-hour incubation, two concentration of each drug were selected. Cells were preincubated with Aroclor1254 (10 and 20 ng/mL) or curcumin (1.5 and 3 µg/mL); and treated for 24 h with vice-versa drugs.
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Preincubation of curcumin at 3 µg/mL was found to inhibit the proliferative effects of Aroclor 1254 at 10 ng/mL where the cytotoxicity values increased to 76.44%; meanwhile this effect was not evident for the other concentrations (Tables 4 and 5; Fig. 4).

Anti-proliferative effect
In support of the MTT results, 3 µg/mL curcumin induced 37% cell death while 6 µg/mL induced 63%. Coincubation of 6 µg/mL curcumin and 40 ng/mL Aroclor induced 80% cell death.

DISCUSSION
Several in vitro models were developed to test potential treatment agents for glioblastoma multiforme amongst most malignant tumours of central nervous system. Curcumin, as a highly antioxidant and apoptotic compound, was previously tested in these in vitro models. Gao et al. (2005) tested the effects of curcumin on malignant glioma cell lines U251MG and U87MG and related the cytotoxic effects on TNF-related apoptosis-inducing ligand (TRAIL) mediated apoptotic pathway through the activation of procaspases and
release of cytochrome C from mitochondria in both extrinsic (receptor related) and intrinsic (chemical related) routes suggesting the potential of curcumin in TRAIL-mediated immunotherapy of malignant gliomas. The effects of curcumin at 25–50 µM concentrations comprised inhibition of the antiapoptotic factors, increase in calpain (natural calcium dependent cystin protease) and caspase expression through both receptor and mitochondrial pathways on human glioblastoma cells, T98G (Karmakar et al., 2006) and U87 MG (Karmakar et al., 2007).

**Table 4.** Effects of curcumin on Araclor 1254-preincubated cells for 24 hours (percent cytotoxicity, mean±SD; n=9)

<table>
<thead>
<tr>
<th>Araclor 1254 (ng/mL)</th>
<th>Curcumin (µg/mL)</th>
<th>Percent cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>48.628±15.523</td>
</tr>
<tr>
<td>3.0</td>
<td>3.0</td>
<td>32.411±6.063</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>45.497±12.965</td>
</tr>
<tr>
<td>20</td>
<td>3.0</td>
<td>34.887±5.654</td>
</tr>
</tbody>
</table>

**Table 5.** Effects of Araclor 1254 on curcumin-preincubated cells for 24 hours (percent cytotoxicity, mean±SD; n=9)

<table>
<thead>
<tr>
<th>Curcumin (µg/mL)</th>
<th>Araclor 1254 (ng/mL)</th>
<th>Percent cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>10</td>
<td>46.821±2.249</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>48.153±2.141</td>
</tr>
<tr>
<td>3.0</td>
<td>10</td>
<td>76.436±6.373</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>34.427±1.011</td>
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</table>

Curcumin was also found to be effective on human and rat glioblastoma cells, T98G, T67 and C6 lines, related mainly on the downregulation of nuclear factor kappa beta transcriptional factor like cancer activators which are important in tumourigenesis (Dhandapani et al., 2007); suggesting curcumin as a promising therapy in malignant brain tumours (Lee et al., 2013). In primary rat cortical neuronal cultures, curcumin at 1–10 µM was found to inhibit the cortical neuronal death and to downregulate activated caspase 3 protein expression (Wang et al., 2012). Curcumin was found to inhibit neuroglial cell growth, to induce glial cellular differentiation through modulation of genes related...
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Fig. 4. F98 cells pretreated with curcumin. A) Karyopyknosis (arrow) of a neoplastic glia cell, curcumin at 1.5 µg/mL; B) Karyopyknosis and cytoplasmic shrinkage (arrows) of neoplastic glia cells, curcumin at 3 µg/mL; C) Karyopyknosis (arrow) of a neoplastic glia cell, co-administration of 1.5 µg/mL curcumin and 10 ng/mL Aroclor 1254; D) Karyopyknosis (arrows) and nuclear blebbings of neoplastic glia cells, co-administration of 3 µg/mL curcumin and 20 ng/mL Aroclor 1254; E) Degenerated neoplastic cell condensations (arrows), co-administration of 3 µg/mL curcumin and 10 ng/mL Aroclor 1254; F) Degenerated neoplastic cell condensations (arrows), co-administration of 3 µg/mL curcumin and 20 ng/mL Aroclor 1254.

to oxidative stress, apoptosis, inflammation, transcriptional regulation, DNA replication and cellular morphogenesis in C6 rat glioma cell line at 5 µM (Panchal et al., 2007); while at 25 to 50 µM concentrations, it inhibited cellular migration by 14.7–53.4% (Kratzer et al., 2012). Side population (SP) phenotyped cells were found to be inhibited by curcumin in C6 cell lines up to 20 times (15 µM for 3 days) (Fong et al., 2010).

Preincubation with Aroclor1254 at 10 ng/mL (30.63 µM) and 20 ng/mL (61.27 µM) was found to induce higher cytotoxicity compared to coinubcation groups with curcumin treatment at 3 µg/mL (8.14 µM); while this effect were found to be lower at lower concentrations (1.5 ng/mL or 4.07 µM). In curcumin preincubated groups in the current study, the most effective was 3 µg/mL (8.14 µM) curcumin group with low Aroclor 1254 concentration (10 ng/mL) (P<0.05). Kratzer (2012) found that curcumin treatment at 25 µM and 50 µM decreased cell counts in migration assay by 14.7% and 53.4% respectively. In the current study, IC₅₀ values were in accordance with both mentioned studies. There are limited studies regarding the effects of Aroclor1254 on glial cells. Madia et al. (2004) found IC₅₀ of Aroclor 1254 on astroglial cells (human 132-1N1 astrociotms) of 26.5±8.04 µM; while in the current study found value was by around 10 ng/mL higher indicating a cell based difference (30.63 µM).

In order to study the effects of curcumin, rat cell lines (with different phenotypes such as astrocytoma and gliosarco-
mas, but mainly glioblastomas) used as in vitro models for both neurogenerative disorders and tumours, are mainly produced through mutagenesis by nitrosourea derivatives. Examples are C6 rat glioma cells and 9L gliosarcoma cells derived from N-methylnitrosourea treated Wistar and Fisher rats and RG2 cells derived from N-ethyl-N-nitrosourea treated Fisher rats. Both F98 (derived from ethylnitrosourea treated rats) and RG2 cell lines are used as a model for human glioma studies due to their invasive characteristic, cell growth and morphological properties (Sibenaller et al., 2005). Meanwhile, due to immunoeffector cells as tumour infiltrating properties lead to false positive results, C6 cells are not favoured for evaluation of potential antitumourogenic drugs (Barth & Kaur, 2009). Therefore, the difference in the effects of curcumin among these studies could be attributed to the difference of cellular defence mechanisms.

The need for alternative and less toxic therapies for head and neck squamous cell carcinoma (HNSCC) is obvious. Multiple molecular pathways such as nuclear factor kappa B (NF-κB) activation, epidermal growth factor receptor (EGFR) and the phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signalling pathways (PI3/AKT/mTOR signalling), signal transducer and activator of transcription 3 (STAT3) expression, the mitogen-activated protein kinase (MAP kinase) cascade and vascular endothelial growth factor (VEGF)-mediated angiogenesis have been shown to be deregulated in HNSCC and represent potential therapeutic targets. While some promising results from such targeted therapies have been obtained, the complexity of interaction between these signalling pathways may contribute to the limited clinical response seen with the use of single-agent biologic therapies. As a natural product, curcumin is both nontoxic as well as diversified in its inhibitory effects on a multitude of pathways involved in carcinogenesis and tumour formation. While the compound alone has shown some anti-tumour effects in HNSCC, curcumin’s lack of systemic toxicity and broad-reaching mechanism of action may make it best suited as an adjuvant therapy for head and neck cancers that are resistant to currently available therapies.

To conclude, curcumin had both anti-proliferative effect on glioblastoma cells; and potential therapeutic effect by its protective role on combinatively PCB (Aroclor 1254) treated cells. It could be concluded that preincubation with curcumin was more efficient when compared to co-incubation; coincubation for 24 hours was more potent when compared to 4-hour treatment; all this indicating that curcumin protects the cells from the deleterious effects of PCB. More studies are required to confirm its effects in terms of mechanism of action and combination with chemotherapeutics.

ACKNOWLEDGEMENTS

This study was supported by funding received from the Scientific and Technological Research Projects Funding Program (TUBITAK) under project number 214O124.

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Paper received 28.09.2017; accepted for publication 30.11.2017

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