Anti-breast cancer activity of curcumin on the human oxidation-resistant cells ZR-75-1 with γ-glutamyltranspeptidase inhibition†

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Since curcumin, a polyphenol extracted from the rhizomes of Curcuma longa L. (Zingiberaceae), has been proposed for breast cancer chemoprevention, the aim of the present work was to determine if it had anti-tumour effects on mammary cells which are resistant to oxidative damage. ZR-75-1 cells were treated with curcumin and copper(II) sulphate in order to evaluate cell death and γ-glutamyltranspeptidase (GGTP) activity. Curcumin was cytotoxic in a dose-dependent manner (loss of viability with lactate-dehydrogenase release) with apoptotic effects on ZR-75-1 cells. Also, curcumin displayed an antioxidant effect only on the copper-oxidized cells. The GGTP activity was decreased in a dose-dependent manner by curcumin, with the changes in this parameter accounting for neoplastic inhibition (direct relation between the enzyme activity and cellular viability). Summing up, our results suggest that curcumin induced apoptosis in ZR-75-1 with an antioxidant activity performed on those treated with copper(II) sulphate, which should be explored more thoroughly with the involvement of the GGTP enzyme activity as biomarker of their malignancy.

Key words: Apoptosis, biomarker, breast cancer, curcumin, copper, γ-glutamyltranspeptidase, oxidative stress

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INTRODUCTION

Breast cancer is one of the most frequent causes of female death worldwide, with its pharmacological treatment being a medical challenge due to its malignancy (1). Regarding this, curcuminoids have been proposed as potential anti-tumour agents for refractory cases (2,3). Thus, the effects of curcumin, a polyphenolic antioxidant extracted from the rhizomes of Curcuma longa L. (Zingiberaceae), need to be evaluated in human aggressive cancer cells, such as the line ZR-75-1.

With regard to preventive strategies, the use of dietary antioxidants has been proposed to counteract the damages induced by oxidative stress (4). Several studies have been directed towards the evaluation of biomedical properties of different plant biomolecules which might be nutraceutical (5). Concerning the potential of these substances, one of the most pursued is the anti-tumour activity acting at different carcinogenic stages (6). From many phytochemicals, the curcumin was proposed as a chemopreventive and chemotherapeutic agent in breast cancer (7). Related to this, curcumin could inhibit cancer initiation, promotion, progression and dissemination in animal models (8). However, given its antioxidant activity, the effects
may differ depending on the cellular redox state. Consequently, in vitro probes should be used in cells challenged with an oxidant agent, such as copper(II) sulphate. Also the xenobiotics (i.e. curcumin and cupric salts), which form part of human diet, could modulate different cellular parameters such as the membrane \(\gamma\)-glutamyltranspeptidase (GGTP), which is involved in cellular antioxidant defence (9). Therefore, GGTP modulation could be considered in oncological interventions, as it may have an active role in cellular proliferation and malignant behaviour (10).

The aim of the present work was to determine the curcumin anti-tumour effect on ZR-75-1 cells, which can resist usual oxidative therapy, by assessing cell death and GGTP activity.

**MATERIALS AND METHODS**

**Chemicals**

Curcumin (CAS n° 458-37-7, \([HOC_6H_3(OCH_3)CH=CHCO]_2CH_2\), MW=368.38 g/mol) and copper(II) sulphate (CAS n° 7758-98-7, CuSO\(_4\), MW=159.61 g/mole) were obtained from Sigma-Aldrich Inc. (USA). The kits for in vitro enzymatic determinations (\(\gamma\)-glutamyltranspeptidase -GGTP-, lactate-dehydrogenase -LDH-) were purchased from Wiener Lab (Argentina). Staining chemicals (Hoechst 33342, methylthiazolyldiphenyl-tetrazolium bromide -MTT-, \(N,N',N''\)-tetramethyl-p-phenylenediamine1,4, dihydrochloride -TMPD-), culture reagents and other substances were obtained from Sigma-Aldrich Inc. (USA).

**Cell culture and treatments**

The human breast cancer cell line ZR-75-1 (American Type Culture Collection) was cultured in RPMI-1640 completed with 10% foetal bovine serum (FBS), 100 U/mL penicillin G and 40 µg/mL gentamycin sulphate, incubated at 37°C in a 5% CO2 atmosphere. After 24 h post-seeding in 96-well plates (30,000 cells/well), cells were incubated for 24 h in medium containing curcumin (0.5, 10, 20 and 40 µM), dissolved in dimethylsulphoxide (DMSO, at a final concentration below 0.05% in the media) with cupric sulphate (0.25 and 10 µM, dissolved in water). The dose range of curcumin used for experiments were chosen in accordance to other researchers in other to modulate molecular targets of malignant development (11), while the copper concentrations were those required for in vitro protein oxidation (12).

**Cellular viability assessment**

After treatment and discarding the media, viable cells were cultured with 60 µL MTT (0.25% in culture media without phenol red) for 4 h. After washing with PBS, the stained cells were solubilized with 100 µL Triton X-100 (10%, 20 min). Results were recorded using a Bio-Rad 680 microplate reader and the relative absorbance was calculated (percentage with respect to Control) at 540 nm (13).

**LDH activity measurement**

Technical requirements were first established to avoid interferences in the study of this enzyme for the experimental conditions of this work. The released LDH by death cells was measured following the LDH-P UV AA kit manufacturer’s instructions, and results (IU/L converted to percentages) were recorded at 340 nm (14).

**Cytological characterization**

Cells were first stained with 1µg/mL of Hoechst 33342 (15 min at 37°C in darkness). After washing three times with PBS, the vital/death phenotype was determined using a fluorescence microscope (Axiovert 100, Zeiss) under UV light (365/380 nm). Images were analysed using the Axio-Vision software (Zeiss) (15).

**GGTP activity measurement**

After the medium was discarded, the active enzyme was released from the cellular membranes using 20 µL of Triton X-100 (10%, 20 min). Then, GGTP was measured following the \(\gamma\)-G-test kinetic AA kit manufacturer’s instructions, adapted to determinations in cultured cells (10). Proteins were measured in 10 µL of the samples by the Bradford method (16), in order to report results as mIU/mg of protein (specific activity).

**Free radical detection**

After the treatments, plates were washed three times with PBS, and cells were lysed with 15 µL of sodium dodecylsulphate (1%, 20 min). 5 µL of the samples were separated for protein determination by the Lowry method (17). Samples (10 µL) were mixed with 50 µL of 16 mM TMPD, incubated for 30 min and measured at 540 nm. Given the high susceptibility of TMPD to oxidizing agents (4), it was prepared in DMSO in order
to stabilize solutions and improve the technical efficiency. The presence of reactive oxygen species (ROS) was calculated by a calibration curve, with results being reported as µM of H2O2/mg of proteins.

Statistical analysis

Data were expressed as means ± standard error (SE) from four separate experiments performed in triplicate. ANOVA models were used to evaluate differences among the treatments. For the comparison of means, Tukey tests were used (p<0.01). Associations between different cellular responses were established by the Pearson coefficient. The statistical analyses probes were performed using the InfoStat 2008e.1 software.

RESULTS

Cellular death

The percentage of viable cells respect to controls was significantly decreased by curcumin in a dose-dependent manner (p<0.0001), with non-copper(II) sulphate-related effects present on this variable (Table). In order to confirm cell toxicity, the LDH activity in the culture media was assayed. A strong inverse correlation between this variable and the decreasing cellular viability (Pearson coefficient=-0.70) was found. In a dose-dependent manner, curcumin increased the LDH release from injured cells (p<0.0001). Although this effect was more notable in cells treated with 10 µM copper (II) sulphate, this salt did not modify the enzyme release (Table). Morphologically, cultures with decreased cellular viability exhibited several apoptotic figures (Figure).

Specific GGTP activity and its correlation with cellular viability

In order to establish the value of this parameter as a breast cancer biomarker, it was necessary to decide an appropriate experimental cell density. Regarding this, a number of 30,000 cells per well was chosen after performing experiments on a wide range of seeded cells (10,000-70,000 cells/well). A direct correlation was found between the specific GGTP activity and the cellular viability assessed by the MTT assay (Pearson coefficient=0.68). Concerning this, curcumin was able to diminish the enzymatic activity dose-dependently, which was enhanced by incorporation of copper (II) sulphate (p<0.0001); (Table).

Free radical level

The cellular oxidative level was significantly increased by copper (II) sulphate at both concentrations (2.5 and 10 µM). Curcumin behaved as an antioxidant agent under copper-related oxidative stress (p<0.0001), with effects being clearer in cells treated with 10 µM of copper (II) sulphate, and absent in copper-unexposed cells (Table).

**Figure.** Cytofluorescence (Hoechst 33342 staining, 400x): ZR-75-1 cells were incubated for 24 h with curcumin at different concentrations (0.5, 10, 20 and 40 µM). The images were representative of those from four separate experiments, with mitotic figures being found in non-treated cells (a), whereas curcumin-treated cells showed several apoptotic figures (b-d).
Table. In vitro parameters in ZR-75-1 cells treated with curcumin and copper

### Cellular viability (% respect to controls):

<table>
<thead>
<tr>
<th>Copper(II) sulphate (µM)</th>
<th>0</th>
<th>2.5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>5</td>
<td>102.94 ± 2.94</td>
<td>110.00 ± 3.33</td>
<td>103.33 ± 3.33</td>
</tr>
<tr>
<td>10</td>
<td>85.29 ± 5.88</td>
<td>76.67 ± 3.33</td>
<td>73.33 ± 6.67*</td>
</tr>
<tr>
<td>20</td>
<td>38.24 ± 2.94*</td>
<td>40.00 ± 3.33*</td>
<td>40.00 ± 3.33*</td>
</tr>
<tr>
<td>40</td>
<td>20.59 ± 0.68*</td>
<td>26.67 ± 3.33*</td>
<td>20.00 ± 3.33*</td>
</tr>
</tbody>
</table>

### Lactate-dehydrogenase release (% respect to controls):

<table>
<thead>
<tr>
<th>Copper(II) sulphate (µM)</th>
<th>0</th>
<th>2.5</th>
<th>10</th>
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<tbody>
<tr>
<td>Curcumin (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>5</td>
<td>108.29 ± 15.94</td>
<td>85.87 ± 5.40</td>
<td>215.18 ± 34.76</td>
</tr>
<tr>
<td>10</td>
<td>152.76 ± 24.48</td>
<td>124.12 ± 24.45</td>
<td>182.50 ± 27.69</td>
</tr>
<tr>
<td>20</td>
<td>242.55 ± 48.96*</td>
<td>167.20 ± 21.91</td>
<td>547.62 ± 56.08*</td>
</tr>
<tr>
<td>40</td>
<td>216.63 ± 22.11*</td>
<td>153.65 ± 23.48</td>
<td>508.57 ± 82.85*</td>
</tr>
</tbody>
</table>

### Specific γ-glutamyltranspeptidase activity (mIU/mg of protein):

<table>
<thead>
<tr>
<th>Copper(II) sulphate (µM)</th>
<th>0</th>
<th>2.5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.87 ± 0.81</td>
<td>6.27 ± 0.62</td>
<td>3.82 ± 0.58*</td>
</tr>
<tr>
<td>5</td>
<td>3.21 ± 0.34*</td>
<td>3.44 ± 0.33*</td>
<td>1.62 ± 0.17*</td>
</tr>
<tr>
<td>10</td>
<td>1.63 ± 0.37*</td>
<td>1.51 ± 0.59*</td>
<td>0.40 ± 0.15*</td>
</tr>
<tr>
<td>20</td>
<td>0.21 ± 0.05*</td>
<td>0.29 ± 0.16*</td>
<td>0.06 ± 0.04*</td>
</tr>
<tr>
<td>40</td>
<td>1.00 ± 0.26*</td>
<td>0.34 ± 0.06*</td>
<td>0.46 ± 0.14*</td>
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</table>

### H2O2 formation (µM/mg of protein):

<table>
<thead>
<tr>
<th>Copper(II) sulphate (µM)</th>
<th>0</th>
<th>2.5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>69.42 ± 5.92</td>
<td>206.14 ± 18.70*</td>
<td>204.38 ± 16.84*</td>
</tr>
<tr>
<td>5</td>
<td>53.73 ± 1.14</td>
<td>60.23 ± 3.63</td>
<td>116.31 ± 15.27</td>
</tr>
<tr>
<td>10</td>
<td>69.72 ± 9.06</td>
<td>70.73 ± 8.47</td>
<td>88.36 ± 15.07</td>
</tr>
<tr>
<td>20</td>
<td>36.69 ± 2.45</td>
<td>79.43 ± 10.37</td>
<td>32.37 ± 13.08</td>
</tr>
<tr>
<td>40</td>
<td>101.01 ± 9.12</td>
<td>115.33 ± 12.69</td>
<td>48.36 ± 4.74</td>
</tr>
</tbody>
</table>

Data were expressed as means ± SE of four separate experiments (*p<0.01).
DISCUSSION

Dietary polyphenols, such as curcumin, could be considered for cancer chemoprevention, which can be primary (preventing illness appearance) and/or secondary (preventing illness progression), and for therapeutic schemes (chemotherapy and chemoadjutancy) (18). In the present study, curcumin was found to be cytotoxic for ZR-75-1 cells in a dose-dependent manner, despite the fact that it retained its antioxidant activity on copper-oxidized cells. These results indicate that cell death was induced by mechanisms different from oxidative damage (19), with the role of thiol-reactive metal ions requiring further studies due to the existence of contradictory data (20). Nonetheless it was previously established that curcumin induced apoptosis on the human breast cancer line MCF-7 (21), the ZR-75-1 cell line was used because it represents a clear example of cells which can tolerate traditional anticancer oxidative-stress-based treatments. Moreover, the viability of this cell line was not compromised by copper-induced peroxide formation. This finding show that these cells resist different kinds of oxidizing agents, such as arsenic and other xenobiotics reported by Soria et al. (10).

Concerning curcumin molecular targets (22), an incipient theory sustains that neoplastic cells have deregulated pathways triggered during cancer initiation and promotion, which are essential for tumour development, such as the GGTP activity. As a result, their inhibition by antioxidants may lead to cellular death (23,24). Also, curcumin can activate the steroid/xenobiotic receptor, which is antiproliferative in breast cancer cells, including ZR-75-1 (25). Regarding this, given the indirect relation between cellular viability and LDH release, we conclude that curcumin was cytotoxic for ZR-75-1 cells leading them to acquire apoptotic features.

Related to cancer development and pathologically-activated antioxidant defences, the GGTP activity has been proposed as a tumour biomarker due to its cytoprotective and pro-proliferating activities (26). Moreover, it was shown to be a sensitive variable with respect to culture conditions (i.e. cellular density), with a strong direct relation being found between the enzyme activity and ZR-75-1 viability, thus supporting its biological role in malignant behaviour. Furthermore, curcumin decreased the GGTP activity (firstly described finding) and compromised the tumour viability. In this regard, the impairment of this antioxidant enzyme may lead to cell death by down-regulation of enzyme-related pro-tumour pathways (27,28). In this regard, it is important to keep in mind that this parameter depends on the cytode differentiation grade for several dietary compounds, showing a direct relation with the viability of undifferentiated cells (i.e. ZR-75-1), whereas other more differentiated types (i.e. MCF-7) exhibit an inverse relation (10; Quiroga et al., unpublished data), with further studies involving a wide range of tumour cell types being encouraged.

CONCLUSIONS

Summing up, the dietary antioxidant curcumin reduced the viability of ZR-75-1 cells, with GGTP being an appropriate biomarker for evaluating the cancer cytotoxic response. In consequence, there is evidence to encourage the use of curcumin as an effective phytochemical in combating tumour growth under conditions of oxidative stress (such in case of the traditional breast chemotherapy) involving an apoptosis-like cell death.

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REFERENCES


