Extended treatment with physiologic concentrations of dietary phytochemicals results in altered gene expression, reduced growth, and apoptosis of cancer cells

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Abstract
Dietary phytochemicals exhibit chemopreventive potential in vivo through persistent low-dose exposures, whereas mechanistic in vitro studies with these agents generally use a high-dose single treatment. Because the latter approach is not representative of an in vivo steady state, we investigated antitumor activity of curcumin, 3,3′-diindolylmethane (DIM), epigallocatechin gallate (EGCG), genistein, or indole-3-carbinol (I3C) in breast cancer MDA-MB-231 cells, exposed in long-term culture to low concentrations, achievable in vivo. Curcumin and EGCG increased cell doubling time. Curcumin, EGCG, and I3C inhibited clonogenic growth by 55% to 60% and induced 1.5- to 2-fold higher levels of the basal caspase-3/7 activity. No changes in expression of cell cycle–related proteins or survivin were found; however, I3C reduced epidermal growth factor receptor expression, contributing to apoptosis. Because some phytochemicals are shown to inhibit DNA and histone modification, modulation of expression by the agents in a set of genes (cadherin-11, p21Cip1, urokinase-type plasminogen activator, and interleukin-6) was compared with changes induced by inhibitors of DNA methylation or histone deacetylation. The phytochemicals modified protein and/or RNA expression of these genes, with EGCG eliciting the least and DIM the most changes in gene expression. DIM and curcumin decreased cadherin-11 and increased urokinase-type plasminogen activator levels correlated with increased cell motility. Curcumin, DIM, EGCG, and genistein reduced cell sensitivity to radiation-induced DNA damage without affecting DNA repair. This model has revealed that apoptosis and not arrest is likely to be responsible for growth inhibition. It also implicated new molecular targets and activities of the agents under conditions relevant to human exposure. [Mol Cancer Ther 2007;6(11):3071–9]

Introduction
Epidemiologic studies indicate that consumption of vegetables, containing dietary phytochemicals, reduces cancer risk (1). Many dietary phytochemicals not only block development of tumors but also inhibit metastatic growth in animal models (Supplementary Table S1). Potential use of dietary agents in combination therapies has been considered among novel treatment approaches because combining phytochemicals with radiotherapy and chemotherapy improves outcome in animal models (Supplementary Table S1).

The majority of studies on these agents in cell culture use short exposure times to high concentrations, often orders of magnitude greater than those achievable in vivo. Moreover, treatment with a single dose provides data on an acute induction response, whereas in vivo anticancer activity arises from a steady-state response to the continued presence of dietary agents. Therefore, many reported effects obtained in cell culture studies may be irrelevant for in vivo activity. All the chemopreventive phytochemicals induce cell cycle arrest and apoptosis in a variety of cancer cells; albeit often at nonphysiologic doses (2, 3). Therefore, we investigated whether any positive antitumorigenic effects (e.g., growth, apoptosis, and expression of selected biomarkers) could be detected following extended treatments with low doses. Furthermore, some of the agents inhibit DNA topoisomerases, DNA methyltransferase, and histone-modifying enzymes (Supplementary Table S2), thereby causing alterations in chromatin packaging and gene expression, which may also modulate response to DNA damage. Hence, we also investigated effect of the phytochemicals on cell response to radiation-induced DNA damage.

We used long-term culturing conditions with concentrations of agents that do not affect viability following a single treatment and are comparable with serum or tissue

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3 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
concentrations achievable in vivo. Concentrations of phytochemicals were 3 μmol/L curcumin, 10 μmol/L 3,5-diindolylmethane (DIM), 8 μmol/L epigallocatechin gallate (EGCG), 2.5 μmol/L genistein, and 30 μmol/L indole-3-carbinol (I3C). Comparable serum concentrations for curcumin (1.77 ± 1.87 μmol/L), DIM (20 μmol/L), EGCG (0.29–7.7 μmol/L), genistein (2.5 ± 1.6 μmol/L), and I3C (28 μmol/L) were detected in humans and/or animal models (4, 5).

Aggressively metastatic breast cancer MDA-MB-231 cells were chosen for this study to investigate whether the phytochemicals had any anticancer therapeutic potential in this model. These cells express estrogen receptor-β, known for particularly high binding affinity to genistein (6), and exhibit a basal-like subtype with epithelial-mesenchymal transition (EMT) to invasive mesenchymal phenotype (7); epithelial genes (e.g., E-cadherin) are significantly down-regulated by DNA methylation in these cells, whereas mesenchymal biomarkers (e.g., vimentin and cadherin-11) or prognostic metastatic biomarkers [e.g., interleukin-6 (IL-6), urokinase-type plasminogen activator (uPA)] are markedly up-regulated.

Materials and Methods

Cell Culture

The human breast cancer cell line MDA-MB-231 was cultured in DMEM with 10% fetal bovine serum as described previously (8). Cells were grown in the presence of DMSO (0.1%) or phytochemicals with fresh medium replenished every 3 to 4 days. In some experiments, MDA-MB-231 cells were treated with 5-aza-2′-deoxycytidine (5-aza-dC; Calbiochem), or trichostatin A (I3C). Comparable serum concentrations for curcumin (2.5 μmol/L), genistein (2.5 μmol/L), EGCG (0.29–1.6 μmol/L), and I3C (28 μmol/L) were presented as mean ± SE.

Cell Growth and Apoptosis

Growth at the end of the long-term treatments was estimated by plating N0 = 10⁵ cells and growing in the medium replenished every 3 days. The number of cells from each treatment was estimated as a percentage of the number in the DMSO control. The number of cell generations (g) was counted according to the formula g = ln2(N/N0), where N is the final number of cells. The time of growth comprises the sum of the time for initial adhesion (considered here to be 5 h), the time of complete division cycles (p × g), and a part of the unfinished last cycle (=p/2 on average), where p is one cell cycle duration. Hence, the time of growth is calculated according to the equation T = 5 + pg + p/2. Therefore, cell cycle duration (p) was estimated as p = (T − 5) / (g + 0.5).

For the clonogenic assay, 300 cells were grown for a further 14 days in the presence of each phytochemical with replenished medium. Colonies were washed, fixed, and stained with crystal violet.

Background apoptotic activity of caspase-3/7 was measured using Caspase-Glo 3/7 kit (Promega) as described previously (9).

Comet Assay

Cells (2 × 10⁵ per vial) in growth medium were irradiated with X-ray doses of 5 and 10 Gy for the DNA damage study. For the repair study, cells were irradiated with 10 Gy followed by incubation in medium at 37°C with 5% CO₂ for 0, 5, 15, and 30 min. Nonirradiated samples were also analyzed. The induction of DNA damage and the comet assay were done as described previously (10). Percentage of tail DNA was measured in two independent cell cultures and data from 200 cells per variant were pooled and presented as mean ± SE.

Quantification of mRNA and Protein Expression

Total RNA isolation, cDNA synthesis, and real-time PCR were done as described previously (8). Assays-on-Demand gene expression kits (Applied Biosystems) were used to quantify mRNA levels for caspase-1, p21Cip1, Bcl-xL, E-cadherin, cadherin-11, uPA, and IL-6 and 18S RNA. Nonmuscle β-actin was quantified using CyberGreen master mix with the oligonucleotides (available on request) in parallel to 18S RNA. The levels of gene expression were calculated by ΔΔC_T method using 18S RNA as a reference.

Analysis of protein expression was done using secondary horseradish peroxidase–conjugated antibody (Dako), detected with enhanced chemiluminescence (Amersham) or enhanced chemiluminescence plus (E-cadherin only), as described previously (8). Alternatively, the secondary antibodies, labeled with fluorescent IR800 and IR680 (Li-Cor) dyes, were detected using with the Odyssey System (Li-Cor). Antibodies used in this study were against epidermal growth factor receptor (EGFR; Biosource), cyclin-dependent kinase 6 (Santa Cruz Biotechnology), cyclin B1 (Novoceastra), cyclin D1 (DakoCytomation), Bcl-xL (BD Biosciences), survivin (Novus Biologicals, Inc.), estrogen receptor-β (Santa Cruz Biotechnology), E-cadherin (Calbiochem), cadherin-11 (Zymed Laboratories, Inc.), and vimentin (BD Pharmingen).

Scratch-Wound Assay

Monolayers of cells, seeded in 12-well plates, were scratched with 20 μL pipette tips and allowed to fill the scratched area for 10 h. Afterwards, cells were fixed and stained. The width of the gap on images was measured using AxioVision software (Carl Zeiss Ltd.).

Data Analysis

Differences among the groups were analyzed using a one-way ANOVA in Statistical Package for the Social Sciences followed by Dunnett’s test to determine whether the treatment groups were different from a control group. P < 0.05 was selected as the level of statistical significance. All data are presented as the mean ± SE.

Results and Discussion

Antitumorigenic Activity

With regard to viability of MDA-MB-231 cells, IC₅₀ concentrations for curcumin, DIM, EGCG, and I3C were 30, 100, 80, and 300 μmol/L (Supplementary Fig. S1), respectively, and are comparable with reported IC₅₀ values in this cell line (9, 11–13). In contrast, genistein reproducibly increased growth in the concentration range 15 to 25 μmol/L.
Likewise, 10 μmol/L genistein enhanced cell growth by 20% in culture and dietary soy supplementation increased breast cell proliferation in patients (14, 15). Published data on genistein are inconsistent, with the IC50 in MDA-MB-231 cells ranging from 10 μmol/L (16) to 120 to 131 μmol/L (17, 18).

Concentrations equal to 10% of the IC50 (i.e., 3 μmol/L curcumin, 10 μmol/L DIM, 8 μmol/L EGCG, and 30 μmol/L I3C) were used for long-term treatment of cells. The concentration of genistein (2.5 μmol/L) was chosen as the maximal dose that did not affect cell number. These

Figure 1. The effect of long-term treatment on cell growth and death. A, cell growth following incubation of 1 × 10^5 for 167 and 194 h after plating is shown as number of cells and cell doubling time. *, *P < 0.05, n = 6. B, results of a clonogenic assay. **, *P < 0.05, n = 6. C, caspase-3/7 activity was measured 30 h after plating the cells. *, *P < 0.05, n = 16.
concentrations did not change cell viability after 48 h (data not shown), in agreement with other reports (2, 14, 19). Cells in passages 10 to 16 growing in the presence of phytochemicals or DMSO, as control, were used for analyses.

Cell growth was estimated after culturing for sufficient time to allow several divisions to facilitate detection of fairly small changes. Curcumin, DIM, and EGCG delayed cell growth by 25% to 30% (Fig. 1A). Curcumin and EGCG increased the doubling time to 36.0 and 35.8 h, respectively, compared with a doubling time of 29.5 h in control cells. The clonogenic assay was used to take account of any influence of autocrine growth factors, which should be significantly decreased by low cell density. Curcumin, EGCG, and I3C decreased the number of colonies by about 2.5- to 3-fold, with markedly smaller size of colonies in the curcumin and I3C groups (Fig. 1B).

Because tumor growth can be hindered by cell death, we investigated whether any of the phytochemicals might affect the basal level of apoptosis. The background caspase-3/7 activity levels were increased in curcumin-treated cells (1.5-fold) and EGCG- and I3C-treated cells (2-fold; Fig. 1C). Interestingly, the basal caspase-3/7 activity levels were inversely related to the colony formation rates in the clonogenic assay (compare Fig. 1B and C).

**Modulation of RNA and Protein Expression**

Treatment with DIM increased mRNA levels of cyclin-dependent kinase inhibitor 1A (p21Cip1) by 11-fold and antiapoptotic Bcl-xL by 3-fold, whereas I3C increased only p21Cip1 mRNA by 3-fold (Fig. 2A). Analysis of protein expression indicated that expression of estrogen receptor-β, cell cycle–related biomarkers (p21Cip1, cyclin-dependent kinase 6, cyclin B1, and cyclin D1), and survival-related proteins (survivin and Bcl-xL) was not changed by treatments,
except for Bcl-xL, which was increased up to 145% in DIM-treated cells (Fig. 2B). p21Cip1 protein was barely detectable. I3C reduced EGFR levels by 48% (Fig. 2B) and vimentin by 35% (Fig. 3A).

Epithelial-mesenchymal transition–related genes, encoding E-cadherin, vimentin, cadherin-11, IL-6, and uPA, were investigated. E-cadherin protein and mRNA levels were very low. Although E-cadherin protein seemed to be up-regulated by curcumin in four of five experiments, no statistically significant increase was found (Fig. 3A and B). Cadherin-11 protein levels were reduced to 78% and 74% by curcumin and DIM, respectively; similar decreases in cadherin-11 RNA levels were detected, although they did not reach statistical significance (Fig. 3A and B). All agents increased IL-6 mRNA levels and all, but EGCG, increased uPA mRNA levels (Fig. 3C). Decreased cadherin-11 and

![Figure 3](image-url)

**Figure 3.** Expression of epithelial-mesenchymal transition biomarkers. **A,** protein levels were analyzed as in Fig. 2. *, $P < 0.05, n = 5$. Levels of mRNA for E-cadherin and cadherin-11 (B) and IL-6 and uPA (C). *, $P < 0.05, n = 4$, analyzed in duplicates. **D,** results of scratch assay with the width measured in μm. *, $P < 0.05, n = 12$, in triplicate.
increased uPA expression may diminish cell-cell adhesion and attachment to the extracellular matrix, which would result in increased cell motility. Hence, cell motility was investigated using the wound assay. Curcumin, DIM, genistein, and EGCG decreased clear areas in the wound assay by 61%, 72%, 85%, and 92%, respectively (Fig. 3D).

Thus, increased cell motility correlated with the increased uPA and decreased cadherin-11 levels in curcumin, DIM, and genistein groups. The cause of increased motility in the EGCG group has not been established.

To investigate regulation of these genes by DNA or histone modification, inhibitors of DNA methylation and histone deacetylase were used. 5-aza-dC significantly increased mRNA expression of IL-6, uPA, p21Cip1, and E-cadherin, whereas cadherin-11 mRNA was decreased; TSA affected gene expression to much lesser extent (Fig. 4A–C). 5-aza-dC also increased protein levels of E-cadherin and vimentin and decreased cadherin-11. TSA similarly affected expression of cadherin-11 (mRNA and protein) and E-cadherin (protein). Up-regulation of p21Cip1, IL-6, and uPA by 5-aza-dC has been shown previously (20–22). Despite a significant increase in p21Cip1 mRNA in 5-aza-dC–treated cells, protein levels were too low to allow reliable detection.

Collectively, these data do not support a proposed role for EGCG in the reactivation of gene expression by inhibiting DNA methylation (23) but agree with the findings of Stresemann et al. (24). Neither did we find significant changes in the histone-3 acetylation levels in the curcumin group (Supplementary Fig. S2),3 which would be expected if curcumin inhibited histone acetyltransferase p300 at physiologic concentrations (25–27). Most of the phytochemicals modulated levels of cadherin-11 and metastatic biomarkers IL-6 and uPA similarly to the inhibitors. This on its own would be an undesirable effect of dietary agents or inhibitors if it were not counterbalanced.

![Figure 4](image.png)

**Figure 4.** Expression of biomarkers in the presence of inhibitors. Cells were treated with 7.5 μmol/L 5-aza-dC or 300 nmol/L TSA for 72 h (A–C) or 2.5 μmol/L PD153035 for 48 h (D). Protein levels were analyzed by immunoblotting with the antibodies indicated. Protein quantification is shown on the graphs as the ratio to actin. *, P < 0.05, n = 3. The levels of mRNA are shown as a fold increase versus DMSO control. **, P < 0.05, n = 3, analyzed in triplicates.
in the chemopreventive net effect. Further studies elucidating roles of these agents in metastatic invasion are required. Compared with the other agents, DIM induced most changes in expression of genes, which can be regulated epigenetically. No evidence of DIM involvement in epigenetic regulation has been reported thus far and further investigation is required.

It was encouraging to note that EGFR was downregulated by I3C in the present study as observed in response to single high doses (8, 9). I3C-induced reduction in vimentin was not related to decreased EGFR signaling (Fig. 4D).

**Response to Radiation**

Radiation-induced DNA damage, detected using the comet assay, was investigated. Some protection against radiation-induced damage (≤16%) was observed in curcumin-, DIM-, EGCG-, and genistein-treated cells (Fig. 5A). DNA repair was not significantly affected (Fig. 5B). Protection from DNA damage was not altered by inhibition of DNA methylation or histone deacetylation (Fig. 5C). DNA damage by X-rays is caused by a burst of free radicals and is likely to be reduced by antioxidant properties of these phytochemicals. Several studies have shown a protective role of dietary phytochemicals in response to ionizing radiation. For example, curcumin protected DNA from chromatid breaks induced by radiation and from chromosome aberrations, DNA damage, and consequent mammary tumors in animal models (28–30). Furthermore, a large body of evidence supports a protective role of dietary phytochemicals in response to UV and oxidative effects (31–35). Moreover, clinical studies show that green tea protects against DNA damage, decreases generation of free radicals, and increases antioxidant activity in healthy volunteers (36).

It is important to note that a protective effect against induced DNA damage can be detected at low concentrations of phytochemicals, which do not induce massive DNA laddering and apoptosis. It has been reported

Table 1. Physiologic activities of dietary phytochemicals in breast cancer MDA-MB-231 cells

<table>
<thead>
<tr>
<th>Effect</th>
<th>Curcumin</th>
<th>DIM</th>
<th>EGCG</th>
<th>Genistein</th>
<th>I3C</th>
<th>TSA</th>
<th>5-aza-dC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling time</td>
<td>↑</td>
<td>≈</td>
<td>≈</td>
<td>≈</td>
<td>≈</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Colony formation</td>
<td>↓, smaller size</td>
<td>≈</td>
<td>↓</td>
<td>≈</td>
<td>↓, smaller size</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
<td>≈</td>
<td>≈</td>
<td>≈</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>DNA damage</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Motility</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RNA levels</td>
<td>↑ IL-6, ↑ p21Cip1, ↑ IL-6, ↑ uPA</td>
<td>↑ IL-6, ↑ p21Cip1, ↑ cadherin-11</td>
<td>↑ IL-6, ↑ uPA</td>
<td>↑ IL-6, ↑ uPA</td>
<td>↑ p21Cip1, ↑ IL-6, ↑ uPA, ↑ E-cadherin, ↓ cadherin-11</td>
<td>↑ p21Cip1, ↑ IL-6, ↑ uPA, ↑ E-cadherin, ↓ cadherin-11</td>
<td></td>
</tr>
<tr>
<td>Protein levels</td>
<td>↓ cadherin-11, ↓ Bcl-xL, ↓ cadherin-11</td>
<td>↓ cadherin-11, ↓ IL-6, ↓ vimentin</td>
<td>↓ cadherin-11, ↓ E-cadherin, ↓ vimentin</td>
<td>↓ cadherin-11, ↓ E-cadherin, ↓ vimentin</td>
<td>↓ cadherin-11, ↓ E-cadherin, ↓ vimentin</td>
<td>↓ cadherin-11, ↓ E-cadherin, ↓ vimentin</td>
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</tr>
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Abbreviation: ND, not determined.

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**Figure 5.** Effect of phytochemicals on DNA damage. A, comet assay was used to detect DNA damage caused by X-ray irradiation (5 and 10 Gy). Damage is expressed as a percentage of DNA in the comet tail. *, P < 0.05 versus DMSO control, n = 200. B, comet assay was also used to detect DNA repair following a 10-Gy X-ray irradiation dose. Time for repair was 5, 15, or 30 min. The repair capacity is expressed as the percentage of the initial damage that is repaired at each incubation time. *, P < 0.05 versus 0 min, n = 200 cells. C, DNA damage in cells treated with 5-aza-dC or TSA was detected as in A.
that higher concentrations of curcumin (15–50 μmol/L; refs. 37, 38) or EGCG (100 μmol/L; ref. 39) induce DNA damage, which is likely to be a result of ongoing apoptosis. Our data suggest that phytochemicals in vitro may protect cells from DNA damage induced by environmental factors and, thereby, slow down their transformation into increasingly malignant phenotypes. However, the data also suggest that dietary phytochemicals may, to a very limited extent, protect cancer cells against radiotherapy.

Comparison of Long-term Treatment with Physiologic Doses with Higher-Dose Single Treatments and In vivo Studies

This in vitro model of long-term exposure to dietary molecules allows the analysis of important activities, summarized in Table 1, at physiologically relevant concentrations. As discussed above, use of higher doses of agents may produce misleading results with regard to DNA damage and in several other respects. Our previous studies showed that treatment of MDA-MB-231 cells with 250 μmol/L I3C decreased protein levels of EGFR, cyclin D1, and cyclin-dependent kinase 6, whereas p21Cip1, p27Kip1, and cyclin E were increased (8). We also showed that I3C-induced apoptosis is related to down-regulation of EGFR in these cells (8, 9). The latter is consistent with the data obtained in this model because reduced EGFR signaling in combination with diluted autocrine transforming growth factor-α is bound to increase apoptosis and reduce growth in clonogenic assay. In addition, in agreement with previous data, I3C increased p21Cip1 RNA (8, 40). However, no changes in cell cycle–related proteins, including p21Cip1, were found in this study. Therefore, data presented here suggest that the main in vivo mechanism of anticancer activity by I3C is likely to be EGFR-related apoptosis rather than cell cycle–related events. This conclusion agrees with the increased apoptosis in tumors of I3C-treated animal models (Supplementary Table S1).3

Similarly, modulation of caspase activity and expression of cell cycle–related proteins, such as p21Cip1, cyclin-dependent kinase 6, cyclin B1, cyclin D1, and antiapoptotic Bcl-xl, have been related to the action of curcumin, EGCG, and DIM, investigated in much higher doses (3): for example, p21Cip1 is increased by high concentrations of genistein (30–74 μmol/L) or EGCG (90 μmol/L; refs. 2, 41, 42). Our data support involvement of apoptosis rather than cell cycle arrest in the action of curcumin and EGCG at physiologic doses in metastatic breast cancer cells. Down-regulation of survivin, proposed as a target in DIM-treated tumors in animal models (Supplementary Table S1),3, was not observed in our study nor was there any change in caspase activity. Up-regulation of p21Cip1 mRNA by DIM, observed in several studies (43, 44), was also detected in our model. However, mRNA levels did not correlate with the protein levels, possibly due to fast modification and degradation of the protein. Importantly, increased apoptosis, induced by curcumin and EGCG, is detected in tumors in animal models (Supplementary Table S1),3, including the MDA-MB-231 xenograft model.

Alltogether, our data show that some phytochemicals had significant antitumorigenic effect on MDA-MB-231 cells, with curcumin and EGCG having the greatest effect on cell viability. The effects of I3C and DIM, a major in vivo acid-catalyzed condensation product of I3C, were different, implying distinct mechanisms of action. Unfortunately, no anticancer effects of genistein were found in this study. Similarly, animal studies did not show inhibition of tumor growth in the MDA-MB-231 cell xenograft by genistein in a serum concentration ~1 μmol/L (45). This correlates with the absence of a protective effect of genistein in estrogen receptor-α–negative tumors (46).

This model has revealed important physiologic activities and new molecular targets and effects (e.g., cell motility or DNA damage protection) of several chemopreventive phytochemicals. It reinforces the view that these agents can exert anticancer activity at physiologically relevant doses and may also be useful for analysis of the effects of dietary phytochemicals on cancer therapies. Future research strategies include investigation into modulation of gene expression and cell motility by dietary agents.

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References


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