

Metabolism of the isoflavones genistein and biochanin A in human breast cancer cell lines¹⁻³

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ABSTRACT There is substantial variation in the growth inhibition of different human breast cancer cell lines by the isoflavones genistein and biochanin A. ZR-75-1 and BT-20 cells are ≥ 2 - to 4-fold less sensitive to these isoflavones than are MCF-7 cells, whereas T47D cells have a sensitivity similar to that of MCF-7 cells. To determine whether these differences are related to isoflavone metabolism by these cancer cells, each of the cell lines was incubated with [4-¹⁴C]genistein and [4-¹⁴C]biochanin A. Metabolites in the cell culture media were identified by radio-HPLC electrospray ionization mass spectrometry. One metabolite of genistein (genistein 7-sulfate) and 2 metabolites of biochanin A (genistein and genistein 7-sulfate) were detected by radio-HPLC. Further analysis by mass spectrometry identified 3 other metabolites, a hydroxylated methylated form of each isoflavone and a biochanin A sulfate. IC₅₀ (the concentration at which the growth rate was halved) values of the breast cancer cell lines did not correlate well with production of genistein 7-sulfate from genistein or with biochanin A sulfate, genistein 7-sulfate, or genistein from biochanin A. However, IC₅₀ values correlated with the production of the hydroxylated and methylated forms of the isoflavones. Only T47D cells produced these metabolites in this study, and only T47D cells had IC₅₀ values similar to those of MCF-7 cells, which also produced the hydroxylated and methylated metabolites. These data suggest that the hydroxylated and methylated metabolites may be the active forms of genistein in human breast cancer cells and emphasize the importance of isoflavone metabolism in the mechanism of action of isoflavones. *Am J Clin Nutr* 1998;68(suppl):1505S–11S.

KEY WORDS Genistein, biochanin A, isoflavones, metabolism, mass spectrometry, breast cancer, cell lines,

INTRODUCTION

Epidemiologic data, preclinical laboratory investigations, and limited clinical trials have indicated that soyfoods are associated with important health benefits because they reduce serum cholesterol concentrations in people with hypercholesterolemia (1–4), prevent osteoporosis in postmenopausal women (5–7), and potentially reduce cancer risk (8). The mechanisms by which the consumption of soy achieves these effects have not been fully elucidated, but may involve several of the phytochemicals in soy (isoflavones, protease inhibitors, phenolic acids, phytosterols, and saponins) (9).

Soy is a unique source of the isoflavones genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone) in the American diet (**Figure 1**). Genistein has been the subject of intense investigation because it is a protein-tyrosine kinase inhibitor *in vitro* (10). It has been shown to inhibit the proliferative effects of several growth factors on both cancer cell and normal cell growth (11–14). Biochanin A (4'-methoxygenistein) is an isoflavone present in large amounts in subterranean clover (**Figure 1**). Although it is a weak protein-tyrosine kinase inhibitor *in vitro* (15), it nonetheless is an inhibitor of growth factor-stimulated cell growth of breast cancer cell lines (14).

Isoflavones are present in soyfoods as β -glucosides (16–18), which are hydrolyzed by gut bacteria to release the highly absorbable aglucones. The unconjugated isoflavones are largely converted to their β -glucuronides in rats by enzymes in the gut wall and the liver (19). After secretion into bile, the isoflavone β -glucuronides are hydrolyzed in the gut and undergo enterohepatic circulation (19).

After ingestion of large amounts of genistein-containing soyfoods, moderate concentrations of both conjugated and unconjugated genistein are found in peripheral blood (20). Genistein may be readily and selectively absorbed by tissues. Evidence for the latter was obtained from women consuming an isolated soy-protein beverage; their nipple-aspirate fluid contained large concentrations of unconjugated genistein, moderate amounts of genistein 7-sulfate, and hardly any genistein β -glucuronide, whereas their blood contained mostly genistein β -glucuronide

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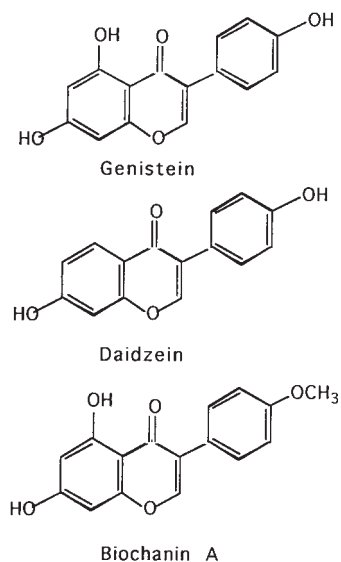


FIGURE 1. Chemical structures of the isoflavones genistein, daidzein, and biochanin A.

(N Petrakis, L Coward, M Kirk, and S Barnes, unpublished observations, 1996).

Genistein inhibits growth factor stimulation of the proliferation of the human breast cancer cell line MCF-7, with an IC_{50} (the concentration at which the growth rate was halved) value of $19 \mu\text{mol/L}$ (11), whereas the IC_{50} value is $3 \mu\text{mol/L}$ for a normal human mammary epithelial cell line (12). The difference in sensitivity to genistein between these cells is due in part to the formation of the 7-*O*-sulfate ester of genistein in MCF-7 cells (21). This metabolite is rapidly excreted from MCF-7 cells, thereby preventing the intracellular accumulation of genistein. Biochanin A, which has an IC_{50} value similar to that of genistein when added to MCF-7 cells (14), undergoes demethylation to form genistein in these cells (21).

Investigations of the inhibitory effects of genistein on other human breast cancer cell lines have shown an even wider range of IC_{50} values (14). Whereas T47D cells (with and without expressed estrogen receptor) have IC_{50} values similar to those of MCF-7 cells, BT-20 cells (estrogen receptor negative) and ZR-75-1 cells (estrogen receptor positive) have IC_{50} values from 46 to $>74 \mu\text{mol/L}$. All these IC_{50} values substantially exceed genistein concentrations in plasma (20). In this study we examined the hypothesis that the sensitivity of breast cancer cells to inhibition of their growth by genistein and biochanin A was related to the extent of metabolism of these isoflavones.

MATERIALS AND METHODS

Materials

The genistin concentrate was 40–50% genistin by weight (Protein Technologies International, St Louis). Fetal bovine serum, tissue culture media, supplements, and antibiotics were obtained from Gibco (Gaithersburg, MD), Clonetics (San Diego), or Upstate Biotechnology (Lake Placid, NY). Tissue culture supplies were from Costar (Charlotte, NC) and biochanin A was from Sigma Chemical Company (St Louis). Sep-Pak C_{18}

cartridges were from Millipore (Bedford, MA), and Aquapore C_8 HPLC columns were from Brownlee Labs (Santa Clara, CA). $[4-^{14}\text{C}]$ Genistein (851 GBq/mol) and $[^{14}\text{C}]$ biochanin A (629 GBq/mol) were custom synthesized by Moravек Biochemicals Incorporated (Brea, CA) and had a radiochemical purity of $>98\%$ by HPLC. All other reagents were of the highest available grade.

Cell culture

BT-20 and ZR-75-1 cells were obtained from the American Type Culture Collection (Rockville, MD). T47D cells were maintained in HEPES-buffered Eagle's modified essential medium with 5% (by vol) fetal bovine serum and antibiotics ($100 \times 10^6 \text{ U penicillin/L}$ and $100 \mu\text{g streptomycin/L}$). BT-20 and ZR-75-1 cells were maintained in RPMI 1640 medium plus 7% (vol:vol) fetal bovine serum and antibiotics as above. All cells were cultured as monolayers (passed every 6–8 d) in a water-saturated atmosphere (95% air:5% CO_2).

Isoflavone isolation

Isoflavones were isolated and prepared from the genistin concentrate as described by Peterson and Barnes (11). The 4'-*O*- and 7-*O*-sulfate ester standards of genistein were synthesized chemically and enzymatically (20, 21).

Metabolism studies

Six-well plates were used to grow cells to 70% confluence. $[4-^{14}\text{C}]$ Biochanin A or $[4-^{14}\text{C}]$ genistein was added to the cells to a final concentration of 3.15 kBq/L and 3.7 or $26 \mu\text{mol/L}$; unlabeled isoflavone was added to adjust the isoflavone concentration. The final dimethyl sulfoxide concentration was 0.5% (by vol). Experiments under each set of conditions were carried out in quadruplicate. Blank wells containing media but no cells were used as controls. After incubation for the indicated times, media were aspirated by suction. Radioactivity of media samples ($100 \mu\text{L}$ portions) was determined by scintillation counting. The remainder of the samples was passed through activated Sep-Pak C_{18} cartridges to collect the $[4-^{14}\text{C}]$ isoflavones and their metabolites. The cartridges were washed with 15 mL distilled water to remove hydrophilic materials. Hydrophobic compounds were eluted with 10 mL 80% aqueous methanol and dried at room temperature with air. Samples were resuspended in $100 \mu\text{L}$ 80% aqueous methanol and analyzed by reversed-phase HPLC.

HPLC analysis

$[4-^{14}\text{C}]$ Genistein and biochanin A metabolites were separated by reversed-phase HPLC with a model 1050 liquid chromatograph (Hewlett-Packard, Palo Alto, CA). Samples were injected onto a 30-cm \times 4.6-mm internal diameter Aquapore C_8 column that was eluted at a flow rate of 1 mL/min with a mobile phase consisting of 0–45% acetonitrile in 0.1% (by vol) aqueous trifluoroacetic acid. Eluted substances were detected by their absorbance at 262 nm. The column eluate was also collected in 30-s fractions and counted to determine the location of radioactive peaks with a model 1900CA liquid scintillation spectrometer (Hewlett-Packard). Data are presented as the means \pm SDs for quadruplicate determinations.

HPLC-electrospray ionization mass spectrometry

Media extracts or individual radioactive genistein metabolite peaks were analyzed by reversed-phase HPLC electrospray ion-

ization mass spectrometry (LC-ESI-MS) with a 10 cm × 2.1 mm internal diameter Aquapore C₈ column that was eluted at a flow rate of 0.2 mL/min with a 0–50% gradient of acetonitrile in 2 mmol ammonium acetate/L. The column eluate was split 1:1 with 100 μL/min going to the electrospray ionization interface of an API III triple quadrupole mass spectrometer (PE-Sciex, Concord, Canada); negative ion mass spectra were recorded by using an orifice potential of –70 V.

RESULTS

The 2 isoflavone concentrations (3.7 and 26 μmol/L) chosen for these experiments corresponded to the IC₅₀ values for genistein in human mammary epithelial cells (12) and in MCF-7 and T47D cells (11, 14), respectively. At genistein and biochanin A concentrations of 3.7 μmol/L, there is no inhibition of serum- or growth factor-stimulated cell proliferation of any of the cancer cell lines tested.

Metabolites of genistein (G1 and G2) and biochanin A (B1, B2, and B3) appearing in the media of each cell line were detected by their absorbance at 262 nm during HPLC analysis. At a genistein concentration of 3.7 μmol/L, there was substantial conversion (89–100%) of genistein (retention time of G2: 9.9 min) into a more polar product (retention time of G1: 8.2 min) in each of the cell lines (Table 1). When incubation was carried out with 26 μmol genistein/L, differences between the metabolites from each of the cell lines could be observed (Table 1 and Figure 2). For ZR-75-1 cells, G1 increased substantially in area and remained the major peak (80% of the total area) (Table 1 and Figure 2A). For both BT-20 and T47D cells, G1 did not increase in area; instead, G2 (genistein) became the predominant peak: being 92 ± 0.5% and 90 ± 1.0% of the total area, respectively (Table 1 and Figure 2, B and C).

As judged by the absorbance profiles, biochanin A (retention time: 11.5 min) was metabolized to several polar products (Figure 3). At 3.7 μmol/L, BT-20 and T47D cells converted biochanin A to B1 (retention time of genistein 7-sulfate: 8.2 min) to 69 ± 2.9% and 48 ± 2.0% of the total area, respectively, and B2 (retention time: 9.9 min) to 31 ± 2.9% and 52 ± 2.0% of the total area, respectively. Only B2 was observed in media extracts of ZR-75-1 cells. B2 appeared to be genistein, but a slight difference in retention time when extracts of media from all 3 cell

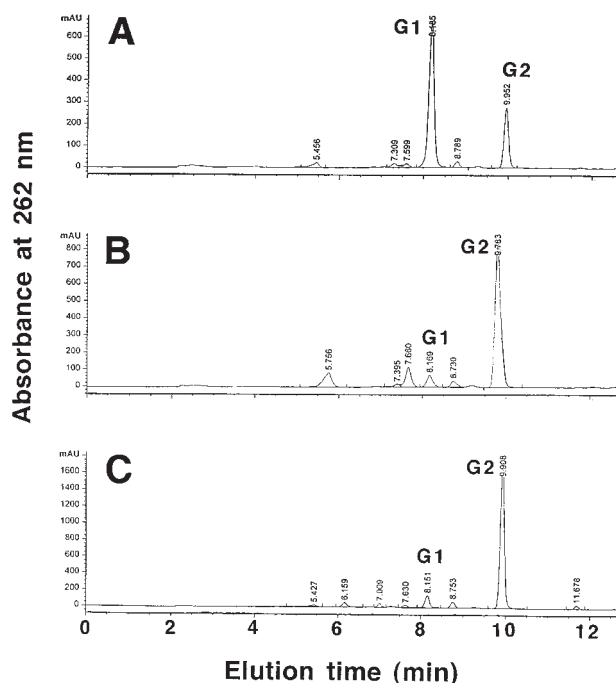


FIGURE 2. Reversed-phase HPLC analysis of genistein and its metabolites in cell media after incubation of 26 μmol genistein/L with human breast cancer cell lines: ZR-75-1 cells (A); BT-20 cells (B); and T47D cells (C). Eluted components were detected by their absorbance at 262 nm. The slight differences in retention times for the genistein peak (G2), eluting at 9.78–9.95 min, resulted because the analyses for each cell line was carried out on separate days over several weeks. G1 was the principal radioactive metabolite of genistein. The unmarked small peaks were due to components in the medium that were unrelated to genistein and its metabolites.

lines were analyzed by HPLC on the same day (data not shown) suggested that it was another metabolite.

When incubation was carried out with 26 μmol biochanin A/L, substantial differences between the metabolites from each of the cell lines were observed. For ZR-75-1 cells, 2 metabolite peaks were present (Table 1 and Figure 3A): B1 (49 ± 0.9%) at 8.2 min, which corresponded to the genistein metabolite G1, and

TABLE 1

Relative proportions of genistein and biochanin and their metabolites in media of cultured cells¹

Cell line and genistein concentration	Genistein peaks		Biochanin A peaks		
	G1	G2	B1	B2	B3
	%		%		
ZR-75-1					
3.7 μmol/L	100 ± 4.8	—	—	100 ± 2.3	—
26 μmol/L	80 ± 5.5	20 ± 5.5	49 ± 0.9	48 ± 0.9	3 ± 0.1
BT-20					
3.7 μmol/L	89 ± 1.0	10 ± 1.0	69 ± 2.9	31 ± 2.9	—
26 μmol/L	8 ± 0.5	92 ± 0.5	—	13 ± 0.9	87 ± 0.9
T47D					
3.7 μmol/L	100 ± 7.2	—	48 ± 2.0	52 ± 2.0	—
26 μmol/L	10 ± 1.0	90 ± 1.0	4 ± 0.2	43 ± 0.6	53 ± 0.6

¹ $\bar{x} \pm SD$. Data are based on the areas of peaks G1, G2, B1, B2, and B3 observed at an absorbance of 262 nm in HPLC analysis of cell media extracts. Each set of values is from data obtained from 4 separate incubations. Because the molar absorbances of each of these peaks are not known and almost certainly not the same, these data are for comparative purposes only.

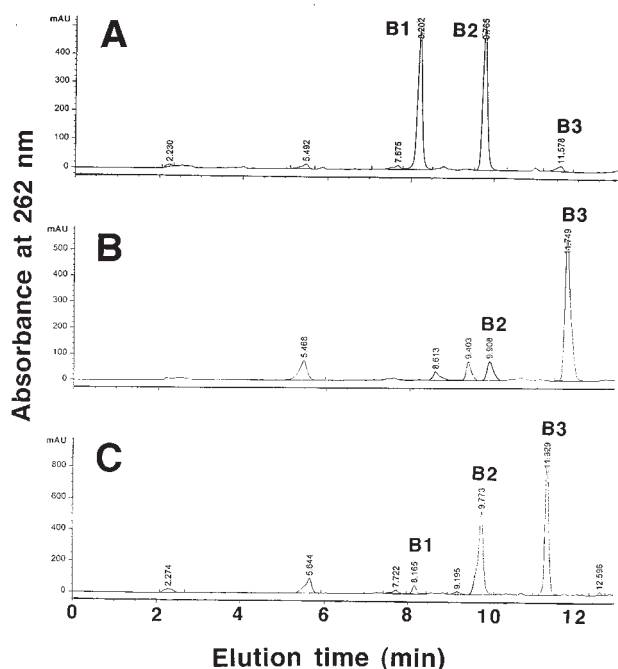


FIGURE 3. Reversed-phase HPLC analysis of biochanin A and its metabolites in cell media after incubation of 26 $\mu\text{mol/L}$ biochanin A/L with human breast cancer cell lines: ZR-75-1 cells (A); BT-20 cells (B); and T47D cells (C). The slight differences in retention times for the biochanin A peak (B3), eluting at 11.33–11.75 min, resulted because the analyses for each cell line were carried out on separate days over several weeks. However, the peak eluting at 9.77 min in the ZR-75-1 cell media was found to elute slightly earlier than genistein. B1 was another radioactive metabolite of biochanin A. Each of the unmarked small peaks was due to components in the medium that were unrelated to genistein and its metabolites.

B2 ($48 \pm 0.9\%$). A small peak of B3 (biochanin A: $3.0 \pm 0.1\%$) was also observed. For BT-20 cells, there were 2 peaks: B2 ($13 \pm 0.9\%$) eluting at 9.9 min as for ZR-75-1 cells and B3 ($87 \pm 0.9\%$) eluting at 11.7 min (corresponding to unreacted biochanin A) (Table 1 and Figure 3B). For T47D cells, 3 peaks were present: a small peak of B1 ($4 \pm 0.2\%$) eluting at 8.2 min, B2 ($43 \pm 0.6\%$) eluting at 9.8 min (corresponding to genistein; however, the peak was broadened by a second unresolved component), and B3 ($53 \pm 0.6\%$) eluting at 11.3 min (corresponding to unreacted biochanin A) (Table 1 and Figure 3C).

Identification of metabolites in human breast cancer cell lines

Verification that the new peaks were derived from genistein and biochanin A was established by measuring the elution of ^{14}C radioactivity after HPLC analysis. ^{14}C radioactivity was associated with each of the new peaks detected by their ultraviolet absorbance. Recovery of ^{14}C radioactivity applied to the HPLC column ranged from 95% to 100%. The slightly longer retention times in these experiments were due to the dead volume of the tubing between the flow cell and the fraction collector. Media extracts were also analyzed by LC-ESI-MS to obtain negative-ion mass spectral data.

ZR-75-1 cells

At an initial genistein concentration of 3.7 $\mu\text{mol/L}$, >95% of the ^{14}C radioactivity in the media supernate after 4 d (Figure 4A)

eluted at 8–8.5 min (G1). LC-ESI-MS analysis confirmed that it was genistein 7-sulfate [mass-to-charge ratio (m/z): 269 and 349]. When the genistein concentration was raised to 26 $\mu\text{mol/L}$, >70% of the ^{14}C radioactivity was genistein 7-sulfate; the remainder was G2, which was confirmed to be genistein (Figure 4B).

Biochanin A, at an initial concentration of 3.7 $\mu\text{mol/L}$, was converted to one more radioactive peak—B2 (Figure 4C). B2 had the same elution time (10 min) as genistein. However, LC-ESI-MS revealed that the genistein peak was biochanin A sulfate (m/z : 283 and 363). Increased abundance of the M+2 isotope ion was observed because it had been labeled with ^{14}C (data not shown). When the biochanin A concentration was raised to 26 $\mu\text{mol/L}$, it was converted to B1 and B2 in approximately equal amounts (Figure 4D). B1 and B2 were identified by LC-ESI-MS as being genistein 7-sulfate and biochanin A sulfate, respectively. A small amount of residual biochanin A was detected.

BT-20 cells

As for the other breast cancer cells, at an initial genistein concentration of 3.7 $\mu\text{mol/L}$, a single more polar metabolite (G1) was formed, accounting for 95% of the ^{14}C radioactivity in the media supernate (Figure 5A). LC-ESI-MS confirmed that it was genistein 7-sulfate (m/z : 269 and 349). An increased ion abundance for ions with an m/z of 271 and 351 could be observed because of the ^{14}C label on this isoflavone. When the genistein concentration was raised to 26 $\mu\text{mol/L}$, the proportion of ^{14}C radioactivity as G1 (genistein 7-sulfate) fell to <10% (Figure 5B).

Biochanin A, at an initial concentration of 3.7 $\mu\text{mol/L}$, was mostly converted to 2 other metabolites: B2, a major metabolite having the apparent retention time of genistein (10.0 min), and B1, a minor metabolite of genistein 7-sulfate with a retention time of 8.2 min (Figure 5C). However, LC-ESI-MS revealed that most of B2 was biochanin A sulfate (m/z : 283 and 363). As before, an increased abundance of ions with an m/z of 285 and 365 could be observed because of the ^{14}C label on this isoflavone

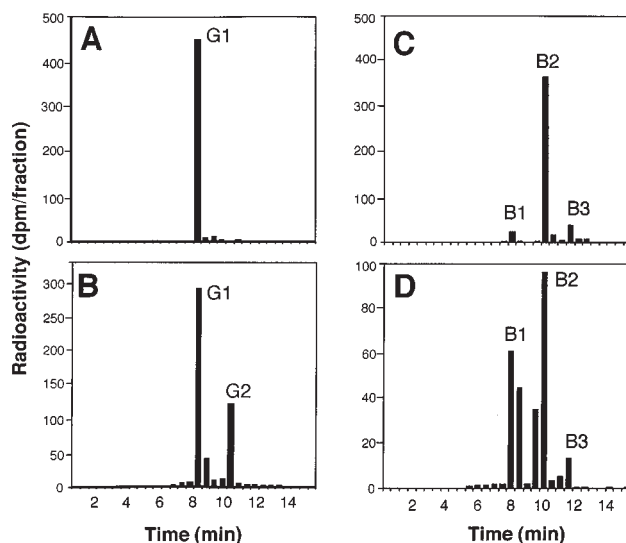


FIGURE 4. Radio-HPLC analysis of the metabolites of genistein (A and B) and biochanin A (C and D) in the cell culture media after incubation with ZR-75-1 cells for 4 d. Two doses were used: the starting concentration was 3.7 $\mu\text{mol/L}$ for A and C and 26 $\mu\text{mol/L}$ for B and D. G1, G2, B1, B2, and B3 correspond to the peaks identified by their ultraviolet absorbance in the HPLC chromatograms in Figures 2 and 3, respectively.

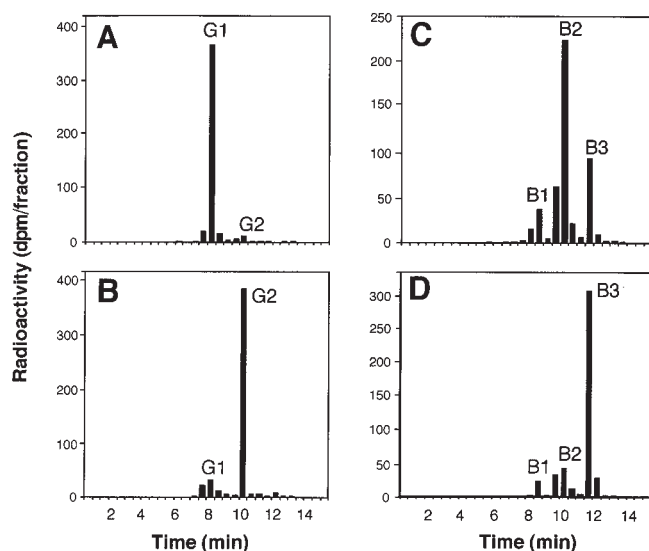


FIGURE 5. Radio-HPLC electrospray ionization mass spectrometric analysis of the metabolites of genistein (A and B) and biochanin A (C and D) in the cell culture media after incubation with BT-20 cells for 4 d. Two doses were used: the starting concentration was 3.7 $\mu\text{mol/L}$ for A and C and 26 $\mu\text{mol/L}$ for B and D. G1, G2, B1, B2, and B3 correspond to the peaks identified by their ultraviolet absorbance in the HPLC chromatograms in Figures 2 and 3, respectively.

(data not shown). Confirmation that B1 was genistein 7-sulfate could not be obtained by LC-ESI-MS analysis (only a m/z 269 ion was observed). When the biochanin A concentration was raised to 26 $\mu\text{mol/L}$, biochanin A (B3) was mostly unreacted (Figure 5D), with $\approx 10\%$ of ^{14}C radioactivity converted to more polar compounds. B2, eluting at 10.0 min, consisted of approximately equal amounts of genistein and biochanin A sulfate, as confirmed by LC-ESI-MS.

T-47D cells

As for the other cell lines, at an initial genistein concentration of 3.7 $\mu\text{mol/L}$, $>95\%$ of the ^{14}C radioactivity in the media supernate after 4 d was G1, eluting at 8.2 min (Figure 6A), and was confirmed by LC-ESI-MS to be genistein 7-sulfate (m/z : 269 and 349); again, an increased abundance of ions with an m/z of 271 and 351 could be observed because of the ^{14}C label on this isoflavone. However, when the genistein concentration was raised to 26 $\mu\text{mol/L}$, the proportion of ^{14}C radioactivity as G1 (genistein 7-sulfate) fell to $\approx 10\%$ (Figure 6B). LC-ESI-MS of the G2 peak revealed that it also contained an ion with an m/z of 299, a hydroxylated and methylated metabolite of genistein observed previously in MCF-7 cells (21). This metabolite was not present in the genistein administered to the cell or in the cell media extracts of the ZR-75-1 or BT-20 cells. As for the other metabolites, LC-ESI-MS revealed that it had an increased abundance of ions with an m/z of 301 because of the ^{14}C label on this isoflavone.

Biochanin A, at an initial concentration of 3.7 $\mu\text{mol/L}$, was almost entirely converted to 2 metabolites (B1 and B2), identified as genistein 7-sulfate and genistein, respectively, with $<5\%$ of biochanin A (B3) remaining unreacted (Figure 6C). However, when the biochanin A concentration was raised to 26 $\mu\text{mol/L}$, $>50\%$ of the ^{14}C radioactivity remained as unreacted biochanin

A (B3) and $<5\%$ was converted to B1 (genistein 7-sulfate); the remainder was B2 (Figure 6D). However, LC-ESI-MS of the B2 peak showed that it not only contained genistein, but also biochanin A sulfate (on the basis of the presence of ions with an m/z of 283 and 363). In addition, the biochanin A and the biochanin A sulfate peaks also contained an ion with an m/z of 313, indicating a hydroxylated and methylated metabolite of biochanin A. For the biochanin A sulfate peak, a molecular ion with an m/z of 393 was also observed. This was analogous to the metabolite observed for genistein. Again, this compound was not present in the biochanin A added to the cells or in the media extracts of the ZR-5-1 or BT-20 cells.

DISCUSSION

This study and a previous report from our laboratory (21) showed that genistein and biochanin A both undergo extensive metabolism in cultured human breast cancer cell lines. In each of these cell lines, genistein is converted in various amounts to its 7-monosulfate. In addition, genistein is metabolized in T47D cells to a hydroxylated and methylated metabolite (Figure 7). Biochanin A is demethylated to genistein, which in turn is also converted to genistein 7-monosulfate. A new finding in the present study is that biochanin A is also converted to its sulfate ester and to a hydroxylated and methylated metabolite, which is also sulfated (Figure 8). This is in contrast with a nontransformed human mammary epithelial cell line that did not significantly metabolize genistein or biochanin A (21) and that was much more sensitive to growth inhibition by genistein (12).

A relation between increased IC_{50} values and the extent of genistein metabolism was not observed at the lower of the 2 concentrations tested (3.7 $\mu\text{mol/L}$) because each cancer cell line converted $>95\%$ of the added genistein to genistein 7-sulfate

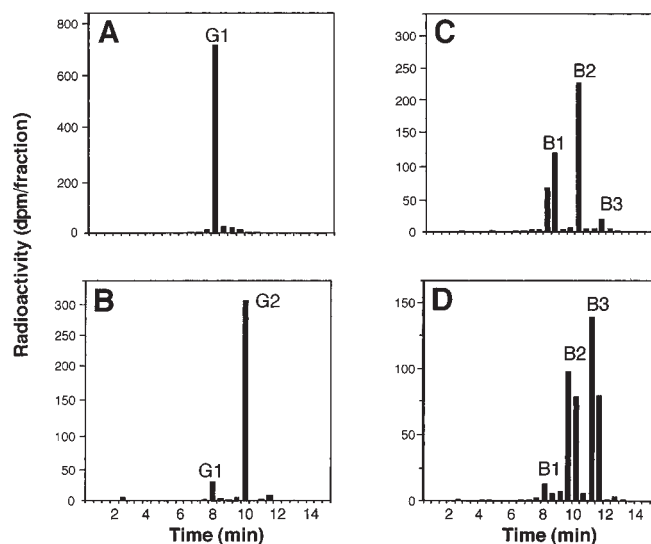


FIGURE 6. Radio-HPLC electrospray ionization mass spectrometric analysis of the metabolites of genistein (A and B) and biochanin A (C and D) in the cell culture media after incubation with T47D cells for 4 d. Two doses were used: starting concentration was 3.7 $\mu\text{mol/L}$ for A and C and 26 $\mu\text{mol/L}$ for B and D. G1, G2, B1, B2, and B3 correspond to the peaks identified by their ultraviolet absorbance in the HPLC chromatograms in Figures 2 and 3, respectively.

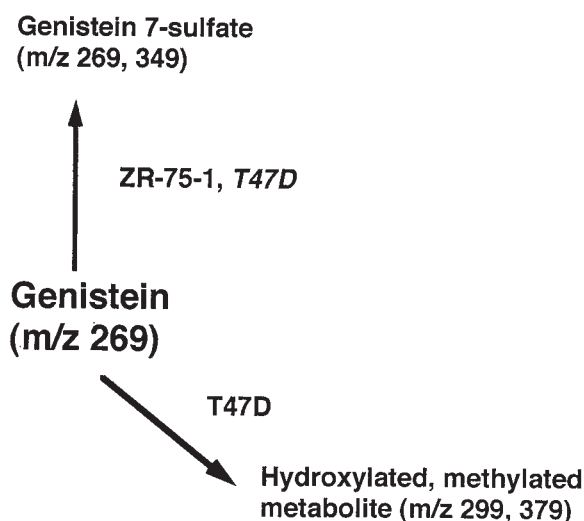


FIGURE 7. Summary of the pathways of metabolism of genistein A in human breast cancer cell lines. Cell lines that are italicized indicate cells in which the pathway is minor. m/z, Mass-to-charge ratio.

over a 4-d period. Interestingly, genistein does not inhibit cell growth of any of the human breast cancer cell lines at these concentrations (14). Indeed, several investigators have reported that when estrogens in the cell media were carefully removed, genistein at concentrations $<5 \mu\text{mol/L}$ stimulated cell growth compared with controls; thus, genistein was classified as an estrogen under these conditions (22, 23). However, data from the present study indicate that genistein may not be the active estrogen, given the extent of its presumed metabolism at these low concentrations.

At the higher genistein concentration ($26 \mu\text{mol/L}$), metabolism of genistein to its 7-sulfate was inversely correlated with the IC_{50} values for genistein. In addition, formation of genistein 7-sulfate was correlated with the previously reported amounts and activities of human phenol sulfotransferase in these cell lines (24). ZR-75-1 cells, which produced the largest amount of genistein 7-sulfate in the media (and hence had the lowest unconjugated genistein concentration), have the highest IC_{50} values ($>74 \mu\text{mol/L}$ for serum stimulation and $52 \mu\text{mol/L}$ for epidermal growth factor stimulation) (14). On the other hand, T47D cells, which have low IC_{50} values (26 and $10 \mu\text{mol/L}$ for serum and epidermal growth factor stimulation, respectively), only converted 10% of the added genistein to genistein 7-sulfate.

The apparent exception to the hypothesis that high IC_{50} values are due to extensive conversion of genistein to genistein 7-sulfate relates to BT-20 cells. This cell line has high IC_{50} values for genistein (although not as high as ZR-75-1 cells) (14), but only converted 10% of the added genistein to genistein 7-sulfate. This did not reflect the relatively high activity of phenol sulfotransferase previously observed in this cell line (24). It was of interest, therefore, that BT-20 and ZR-75-1 cells did not convert genistein to the hydroxylated and methylated metabolite observed here in the T47D cell line and previously in MCF-7 cells (21). It raises the possibility that in human breast cancer cells, genistein may be a prodrug, with the hydroxylated, methylated metabolite as the active form. A potentially similar compound, 2-methoxyestradiol, was shown to have antitumor

and antiangiogenic activity (25). If an analogous metabolite were formed from genistein, it would be 6-methoxygenistein. Interestingly, this compound could also have the trivial name 5-hydroxyglycitein.

A more complex pattern of metabolites was observed for biochanin A. For ZR-75-1 cells, biochanin A at both doses was metabolized to what initially appeared to be substantial amounts of genistein. However, LC-ESI-MS showed that the "genistein" was really biochanin A sulfate. This would explain the marked insensitivity of the ZR-75-1 cells to growth inhibition by these isoflavonoids. These cells, because of their high aryl sulfotransferase activity (21), convert the isoflavones to more readily excretable metabolites, lowering intracellular concentrations of the isoflavones. The site of sulfation of biochanin A was not formally established, but, by analogy with genistein (21), is expected to be at the 7-position. Interestingly, ZR-75-1 cells at a high density also exhibited demethylation of biochanin A with the appearance of genistein 7-sulfate. It is not clear whether demethylation occurred before or after sulfation.

For T47D cells, partial metabolism to genistein, but not to genistein 7-sulfate, occurred at the higher dose, allowing for intracellular accumulation of genistein; this explains their sensitivity to growth inhibition by biochanin A. For BT-20 cells, with growth weakly inhibited by biochanin A (14), metabolism of biochanin A to genistein was low at each dose. As for genistein, biochanin A was also converted to a hydroxylated and methylated metabolite in T47D cells but not in ZR-75-1 and BT-20 cells. The metabolite was present in both the free and sulfated forms.

The preservation of expression of the cell phenotype, and hence the profile of metabolic events, is a recurrent problem in cultured cells. Accordingly, translation of the importance of the results from this study to the *in vivo* situation cannot be fully assessed. Currently, it is not known whether genistein and biochanin A are converted to their sulfate esters in the tumors of patients with breast cancer, although it is expected that sulfation occurs in the human liver because of the known expression of this enzyme in that organ (26). Sulfate esters of isoflavones and their metabolites are present in the plasma (27) and urine (28) of

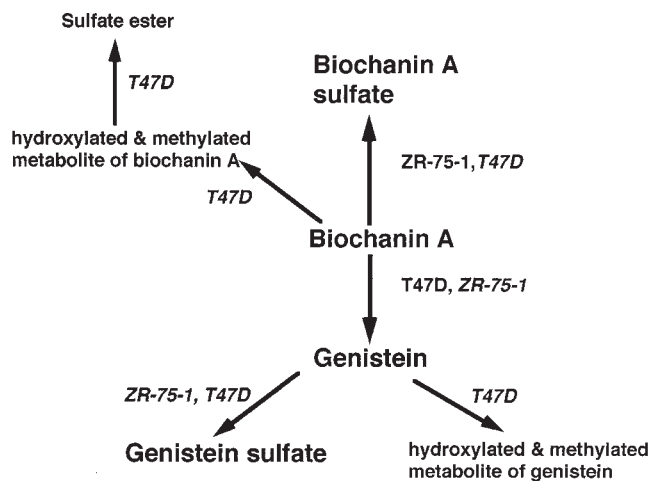



FIGURE 8. Summary of the pathways of metabolism of biochanin A in human breast cancer cell lines. Cell lines that are italicized indicate cells in which the pathway is minor.

soy consumers without disease, although β -glucuronide conjugates usually predominate. Measurements of isoflavones and their metabolites in patients with a breast cancer tumor burden have yet to be reported.

The present findings and those of our previous studies (12, 14, 21) suggest that genistein is more effective in the early stages of breast cancer development, before the breast cancer phenotype is fully established. This is consistent with epidemiologic data (8) and the results from animal models of breast cancer (29–31). Once tumors are established, low circulating concentrations of unconjugated genistein are likely to be metabolized even at the target tissue level. For genistein to be of benefit to patients with frank clinical tumors, much higher doses of genistein than can be provided via dietary sources may be necessary to overcome the hepatic first-pass clearance and the effects of intratumor metabolism. Another alternative, used therapeutically in the case of estrogenic steroids, would be patch technology, in which genistein in its unconjugated form is delivered directly to the tissue target. 

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