Triterpenoid Saponins from *Acacia victoriae* (Bentham) Decrease Tumor Cell Proliferation and Induce Apoptosis

Kalpana Mujoo, Valsala Haridas, Joseph J. Hoffmann, Gerald A. Wächter, Louis K. Hutter, Yiling Lu, Mary E. Blake, Gamini S. Jayatilake, David Bailey, Gordon B. Mills, and Jordan U. Guttermann

INTRODUCTION

Today, about one-fourth of all medications contain an active ingredient derived from a plant. Recently, we began to evaluate extracts of arid climate zone species from the Leguminosae family (1) to identify potential new anticancer compounds. In this report, we describe the isolation and partial purification of novel triterpenoid saponins (F035) from an *Australian desert tree of the Leguminosae family*. F035 and the avicins markedly inhibited the growth of several tumor cell lines with minimum growth inhibition in human foreskin fibroblasts, mouse fibroblasts, and immortalized breast epithelial cells at similar concentrations. F035 and the avicin induced cell cycle arrest in the human MDA-MB-435 breast cancer cell line and apoptosis of the Jurkat (T-cell leukemia) and the MDA-MB-435 breast cancer cell line. The triterpenoid saponins also partially inhibited phosphatidylinositol 3-kinase activity in Jurkat T cells in a time-dependent manner and phosphorylation in the downstream protein Akt, whereas no effect was seen on the Ras/mitogen-activated protein kinase cascade. These observations as well as other work from our laboratory demonstrating mitochondrial perturbation, chemoprevention, and inhibition of nuclear factor kB suggest that triterpenoid saponins from *A. victoriae* have potential as novel anticancer agents. Recent work linking Akt signaling with glucose metabolism, stress resistance, and longevity suggests other potential applications of these compounds.

MATERIALS AND METHODS

**Partial Purification of *A. victoriae* Extracts.** Sixty plant extracts (collected from the Desert Legume Program at the University of Arizona at Tucson, AZ) were prepared by a water-methanol or dichloromethane:methanol extraction at the University of Arizona (Tucson, AZ). One of these extracts exhibited growth inhibitory activity against a panel of tumor cell lines. Preliminary spectral analysis suggested that the *A. victoriae* plant extract contained triterpenoid saponins. A mixture of the triterpenoid saponins was isolated from the aerial parts of the plant as described. It was ground to a 3-mm particle size in a Wiley mill. Then it was packed into a 2-liter percolation unit. The air-dried and ground plant material (aerial parts, 540 g) was then extracted twice with methanol:dichloromethane at room temperature. The solvent was removed in a vacuum, and the solid residue (51 g) was treated with methanol:dichloromethane and rechromatographed. From the resulting fractions, we selected F035 based on the highest yield (2.19 g), and designated it for additional biochemical evaluation. Additional purification of a mixture of triterpene glycosides on C18 reverse-phase semipreparative high-performance liquid chromatography column yielded two components termed avicin D and G.

**Cell Lines.** The following human cell lines were obtained from American Type Tissue Culture Collection (Rockville, MD): SK-OV-3, OVCAR-3, Jurkat, U-937, MDA-MB-468, MDA-MB-453, MDA-MB-435, SK-BR-3, MCF-7, MDA-MB-231, BT-20, LNCaP, PC-3, DU145, 769-P, 786-O, A498, PANC-1, and Hs 27. Mouse fibroblasts L929 were also obtained from ATCC. Cells were grown in medium supplemented with 10% FCS, 200 µM glutamine, and 0.05% gentamicin.

**Growth Inhibition.** Jurkat cells (0.5–1 × 10^6/well) were cultured in 96-well plates with different concentrations of extracts and incubated for 72 h at 37°C. Inhibition of cell growth was assessed by MTT dye conversion (3) or crystal violet staining (4) as described previously.

**Cell Cycle Analysis.** Jurkat cells (1 × 10^6/ml) were treated with various concentrations of the F035 mixture, avicin D or avicin G (0.5–1.0 µg/ml) for 16 h at 37°C. Cells were washed and incubated in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2 mM CaCl_2) containing 1 µg/ml of Annexin V-FITC conjugate (BioWhittaker, Walkersville, MD) for 10 min in the dark. Cells were then stained with propidium iodide (20 µg/ml) and analyzed by flow cytometry (6).

**PI3K Assay.** Jurkat cells were cultured 0.5% serum containing medium and treated with F035, avicins D and G, or wortmannin at 37°C for the indicated times. PI3K was immunoprecipitated from 1 µg of cellular protein, and its activity was determined as described previously (7, 8).

**Western Blotting.** Jurkat cells cultured in 0.5% serum containing medium were treated with F035, avicins D and G (0.5–2.0 µg/ml), or wortmannin (1 µM) for 2–16 h at 37°C. MAPK phosphorylation was stimulated by CD3

Received 10/19/00; accepted 5/11/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by grants from the Clayton Foundation for Research (to M. K., V. H., J. U. G.), the Foundation for Research (to J. H., J. A. W., L. K. H., M. E. B.), and grant CA 64602 (to G. B. M.).

2 To whom requests for reprints should be addressed, at Department of Molecular Therapeutics, Box 41, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-2676; Fax: (713) 792-6554; E-mail: jgutterm@mdanderson.org.

3 The abbreviations used are: F035; Fraction 35; PI3K, phosphatidylinositol 3-kinase; avicins D and G, *Acacia victoriae* triterpenoid saponins; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; zVAD-fmk, z-Val-Ala-Asp-CH₂; P, MAPK, mitogen-activated protein kinase; A. victoriae, *Acacia victoriae*. 

5486
cross-linking for 10 min at 37°C. Levels of total and phosphorylated forms of Akt and MAPK were determined by Western blot analysis using phosphospecific Akt (Ser-473 or Thr-308), a phosphorylation-independent Akt antibody (New England Biolabs, Beverly, MA), or a phosphospecific extracellular signal-regulated kinase antibody (p42 and p44; Promega Corp., Madison, WI).

RESULTS

**F035 and Avicins Inhibit Tumor Cell Proliferation.** As shown in Fig. 1, Jurkat T cells were highly sensitive to F035 with an IC$_{50}$ of 0.2 µg/ml. Similarly, F035 inhibited the growth of a number of cancer cell lines with concentrations inhibiting growth by 50% (IC$_{50}$) in the range of 0.72–6.5 µg/ml. However, a number of breast cancer cell lines were resistant to the inhibitory effect of F035, demonstrating selectivity of response. The last three bars of Fig. 1 show that the IC$_{50}$ for nontransformed cells (mouse fibroblasts and immortalized breast epithelial) was >25 µg/ml, suggesting that F035 inhibits the proliferation of a selected group of cancer cell lines without affecting normal cells significantly.

In addition, avicins D and G were tested for growth inhibitory activity on five cancer cell lines. Fig. 2 shows that avicin D inhibited the growth of a number of cancer cell lines with an IC$_{50}$ that is comparable with F035. However, avicin G (IC$_{50}$ 0.12–1.49 µg/ml) showed significantly more growth inhibitory activity than F035 and avicin D in most of the cell lines tested ($P < 0.05$), which may be explained by the increased hydrophobicity of avicin G.

**F035 and Avicins Induce Cell Cycle Arrest and Apoptosis.** MDA-MB-453 cells treated with F035 showed an increase in the population in G$_1$ phase (7–10%) with a concomitant decrease in the percentage of cells in the S phase (6–10%), suggesting a G$_1$ arrest. In contrast, the number of MDA-MB-435 cells in the sub-G$_0$ phase of cell cycle arrest increased to 16% (Table 1) in response to treatment with F035, suggesting that the cells underwent apoptosis. A terminal
Table 1. Cell cycle analysis of F035-treated cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>F035 µg/ml</th>
<th>Sub G&lt;sub&gt;0&lt;/sub&gt;</th>
<th>G&lt;sub&gt;1&lt;/sub&gt;</th>
<th>S</th>
<th>G&lt;sub&gt;2&lt;/sub&gt;/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-453</td>
<td>0</td>
<td>1.0</td>
<td>62</td>
<td>26</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5</td>
<td>69</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.8</td>
<td>71</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2.2</td>
<td>72</td>
<td>19</td>
<td>9.0</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>0</td>
<td>1.0</td>
<td>52</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.0</td>
<td>51</td>
<td>36</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13</td>
<td>50</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>16</td>
<td>50</td>
<td>26</td>
<td>10</td>
</tr>
</tbody>
</table>

deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay with F035 and the avicins (APO-DIRECT; Phoenix Flow Systems, San Diego, CA; data not shown) confirmed the observation that F035 and the pure avicins induced a cell cycle arrest and/or apoptosis, depending upon the cell line. We assayed for Annexin-V binding in treated Jurkat cells. As shown in Fig. 3, 15–17% of cells treated with 1 µg/ml of F035, avicin D, or avicin G bound to Annexin V-FITC, suggesting that these cells were progressing through early stages of apoptosis. Sixteen to 37% of these cells demonstrated binding to both Annexin V and propidium iodide, suggesting cell death, though this assay does not distinguish between apoptosis and other forms of cell death. Avicin G was ~50% more active in mediating cell death than avicin D or F035.

**F035 and Avicins Inhibit Signaling through the PI3K Pathway.**

The results of immunoprecipitation with a pan-anti-p85 antibody, which coprecipitates the p110 catalytic subunit of PI3K and subsequent lipid kinase assay, showed that F035 and avicins inhibited PI3K activity in Jurkat T cells. Cells treated with 0.5 µg/ml of F035, avicin D, or avicin G inhibited PI3K activity by 60–90%. Wortmannin (1 µM, 16 h after treatment), a known irreversible PI3K inhibitor, was used as a positive control (Fig. 4A). A study of the time course of this inhibition at 2 µg/ml revealed that as early as 2 h after treatment with F035 or avicin D, PI3K activity decreased by 26–31%, whereas a similar treatment with avicin G induced an 83% decline in PI3K activity. In contrast to wortmannin, inhibition of PI3K activity by triterpene glycosides persisted to 16 h, demonstrating that the effects of the compounds on PI3K activity is time-dependent (Fig. 4B).

To examine the effect of the F035 and avicins in the cell-free system, we added F035, avicin D, or avicin G directly to immunoprecipitated PI3K from Jurkat T cells. Avicin G at 100 µg/ml induced a 54% decrease in PI3K activity. At lower concentrations, the effects were less pronounced (Fig. 4C). These results suggest that the compounds may need to be metabolized by whole cells into an active form. Alternatively, they may act on a target upstream of PI3K, resulting in decreased PI3K activity in intact cells. Because treatment of cells with F035, avicin D, avicin G, or wortmannin did not alter p85 (Fig. 4D) and p110 protein levels (data not shown), loss or degradation of p85 and p110 does not explain the decreased PI3K enzyme activity observed in triterpene glycoside-treated cells.

**F035 and Avicins Inhibit Phosphorylation of Akt.** We next examined the effect of F035 and avicins on Akt, a serine threonine kinase and a downstream effector of the PI3K signaling pathway. In
contrast to the rapid and strong inhibition of PI3K activity, inhibition of Akt phosphorylation at Ser-473 and Thr-308 was modest and delayed. Treatment of Jurkat cells with F035 (0.5–1 μM/ml) for 16 h resulted in an inhibition of Akt phosphorylation at Ser-473 (17–34%) and Thr-308 (36%) accompanied by a 19% decrease in total Akt protein (Fig. 5A). A similar treatment with avicin D inhibited Akt phosphorylation at Ser-473 (17–24%) and Thr-308 (35–41%) with no detectable effects on total Akt protein levels (Fig. 5A). However, under similar conditions avicin G reduced Akt phosphorylation at Ser-473 by 50–79% and at Thr-308 by 60–78%. The total Akt protein decreased by 28–57% in response to avicin G (Fig. 5, A and B). These results demonstrate that F035 and avicins induce a time- and dose-dependent inhibition of Akt phosphorylation. Treatment of Jurkat cells with wortmannin showed complete inhibition of Akt phosphorylation at 2 h and ~60% inhibition at 16 h posttreatment, which may be attributable to a shorter half-life of wortmannin (Fig. 5B).

To examine the possibility that degradation of Akt protein during apoptosis could lead to changes in Akt phosphorylation, we pretreated the cells with a broad cell-permeable irreversible pan-caspase inhibitor zVAD-fmk for 1 h at 37°C. This step was followed by treatment of cells with F035 and avicins D and G. Fig. 5C demonstrates that zVAD-fmk did not block the inhibition of Akt phosphorylation at Ser-473 exhibited by F035, avicin D, and avicin G. However, pretreatment of cells with pan-caspase inhibitor zVAD-fmk blocked the degradation of Akt by caspases, thereby suggesting that the decrease in total Akt levels observed in Jurkat T cells may be attributable to apoptosis. These results also suggest that a decrease in Akt levels is insufficient to explain the effects of F035 and avicins on Akt phosphorylation.

**F035 Does Not Inhibit Phosphorylation of MAPK.** MAPK, like PI3K, is activated by multiple growth factors, and this pathway contributes to cell growth, transformation, and viability. Treatment of Jurkat cells with 1–2 μg/ml of F035 for 16 h (a concentration and time sufficient to markedly inhibit signaling through the PI3K pathway) did not have any effect on CD3-induced phosphorylation of MAPK, suggesting that F035 may not target the MAPK pathway (Fig. 5D).

**DISCUSSION**

On the basis of bioassay-directed fractionation using growth inhibition of tumor cells as a monitor, we describe the extraction and partial purification of novel triterpene glycosides from an Australian desert tree called *A. victoriae*. Additional purification of novel avicins has been reported separately (2). Using Annexin V binding assays, we have shown that F035 and avicins were able to induce apoptosis of Jurkat T cells and the MDA-MB-435 breast cancer cell line in a dose-dependent manner. In the MDA-MB-435 breast cancer cell line, F035 and avicins induced cell cycle arrest rather than apoptosis in a dose-dependent manner. The reason for this differential response in different cell lines is currently unknown.

To ascertain the mechanism of action of these triterpenoid saponins, we studied two signaling pathways that lead to increased cell proliferation or cell viability and are crucial for tumorigenesis, namely PI3K and MAPK (9, 10). PI3K, which is activated in response to multiple growth factors, is involved in survival, proliferation, and tumorigenesis (11). Treatment of Jurkat cells with F035 or avicins resulted in a time-dependent inhibition of PI3K activity. We also demonstrated an inhibition in *vitro*, though much higher concentrations of F035 and avicins were required. These results suggest that F035 and avicins could be inducing a post-translational modification of PI3K. Alternatively, these agents could be metabolized by cells to an active form, or their action may require a cellular cofactor similar to the FK506 binding proteins essential for the action of FK506, cyclosporin A, and rapamycin (12). Another possibility is that events upstream of PI3K, such as inhibition of focal adhesion kinase (13), could also be responsible for the inhibition of PI3K by F035 and avicins.

Akt (a cellular homologue of the viral oncogenic protein v-Akt, also known as protein kinase B) is activated by a number of growth factors as a consequence of PI3K activation (14). Akt encodes a serine threonine protein kinase that is amplified in ~15% of ovarian and pancreatic carcinomas (15, 16). In contrast to the 60–90% inhibition of PI3K activity, we observed a partial and delayed inhibition of Akt activity in response to F035 and avicins. These results suggest that there may be other members of the PI3K pathway involved in this phenomenon. In some systems, Akt has been shown to be activated independently of PI3K and MAPK by Ca²⁺/calmodulin-dependent protein kinase kinase because the increase in the intracellular Ca²⁺ concentration promotes survival of some cultured neurons (17).

Whether inhibition of the PI3K/Akt signaling is necessary for cell growth inhibition is not clear. Additional work from our laboratory has revealed that F035 and avicins induce apoptosis in Jurkat cells by directly perturbing the mitochondria while reducing generation of...
reactive oxygen species (2). The kinetics of cytochrome c release followed by caspase activation suggest that the inhibition of the PI3K pathway is probably a secondary event responsible for the proapoptotic function of these agents (2). Yet, even as a secondary event, the biological significance of the interruption of the PI3K pathway may be significant. Recent reports showing the role of Akt in inactivating caspasess (18) suggest that inhibition of the PI3K/Akt pathway could indirectly contribute to the induction of apoptosis.

We have also observed that these agents inhibit tumor necrosis factor-induced activation of nuclear factor-κB (19). The time course of this effect is very similar to that observed with the inhibition of the PI3K pathway. Recently, there have been reports showing the involvement of PI3K/Akt in the activation of nuclear factor-κB (20). Whether the two pathways are related in our system remains to be elucidated. It therefore appears that effects of these triterpenoid saponins on the PI3K pathway, though secondary, could in turn regulate other signaling cascades critical for tumorigenesis.

Data reported here in conjunction with other work from our laboratory (2, 19, 21) clearly suggest that mixtures of triterpenoid saponins and/or avicins could have important potential as cancer preventive and therapeutic agents. Finally, recent work linking Akt signaling with glucose metabolism, stress resistance, and longevity suggests other potential applications of these compounds (22, 23).

ACKNOWLEDGMENTS

We thank Donna Zapatka, Kathy McKee, and Sarah Lock-Lim for excellent technical assistance, Ruth LaPushin for helping with the cell cycle and apoptosis assays, and Dr. Srikanth Reddy for conducting a PI3K assay.

REFERENCES


Triterpenoid Saponins from \textit{Acacia victoriae} (Bentham) Decrease Tumor Cell Proliferation and Induce Apoptosis

Kalpana Mujoo, Valsala Haridas, Joseph J. Hoffmann, et al.


\textbf{Updated version}  Access the most recent version of this article at:  
\url{http://cancerres.aacrjournals.org/content/61/14/5486}

\textbf{Cited articles}  This article cites 19 articles, 5 of which you can access for free at:  
\url{http://cancerres.aacrjournals.org/content/61/14/5486.full#ref-list-1}

\textbf{Citing articles}  This article has been cited by 7 HighWire-hosted articles. Access the articles at:  
\url{http://cancerres.aacrjournals.org/content/61/14/5486.full#related-urls}

\textbf{E-mail alerts}  Sign up to receive free email-alerts related to this article or journal.

\textbf{Reprints and Subscriptions}  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at \url{pubs@aacr.org}.

\textbf{Permissions}  To request permission to re-use all or part of this article, use this link  
\url{http://cancerres.aacrjournals.org/content/61/14/5486}.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.