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Triflavin, an Arg-Gly-Asp-Containing Peptide, Inhibits B16-F10 Mouse Melanoma Cell Adhesion to Matrix Proteins via Direct Binding to Tumor Cells

Key Words
Triflavin
RGD-containing peptide
Extracellular matrix
Melanoma cells

Abstract
Triflavin, an Arg-Gly-Asp (RGD)-containing snake venom peptide, inhibits B16-F10 mouse melanoma cell adhesion to extracellular matrices, e.g., fibronectin, vitronectin, fibrinogen, and collagen type I. In this study, GRGDS inhibits B16-F10 mouse melanoma cell adhesion to immobilized triflavin in a dose-dependent manner. In addition, flow-cytometric analysis and the fluorescence staining method in which FITC-triflavin is utilized as a binding ligand were used. GRGDS inhibits the binding of FITC-triflavin to B16-F10 cells. Additionally, the above results suggest that triflavin directly binds to its receptors expressed on B16-F10 cell surface primarily via its RGD sequence, thereby inhibiting B16-F10 cell adhesion to extracellular matrices.

Metastasis is a complicated phenomenon that involves tumor cell dissemination and interaction of tumor cells with host cells (i.e., platelets, endothelial cells) and subendothelial matrices. Cellular interactions are mediated by cell-surface molecules expressed by tumor cells as well as by host cells. Those surface molecules also mediate cell-cell recognition, adhesion, and interaction with subendothelial matrix components [8]. A family of cell surface adhesion receptors termed 'integrins' has been described elsewhere [19]. Integrins comprise a superfamily of transmembrane receptors that participate in cell-cell and cell-substrata interactions. Integrin receptors are membrane-spanning heterodimers consisting of noncovalently associated α- and β-subunits [9, 10]. Many of the extracellular matrix (ECM) receptors (e.g., the family of VLA antigens) are composed of a common β_1 subunit complexed to one of several α-chains [30]. The multiple ligand-binding capability of those integrins is due to their ability to recognize Arg-Gly-Asp (RGD) sequence [21, 25]. Evidence suggests that tumor cells interact with RGD-containing proteins during invasion. Gehlsen et al. [5] demonstrated that the RGD peptide blocked tumor cell invasion of a basement membrane. In addition, other investigators revealed that RGD or its polymers could inhibit experimental tumor metastasis [18, 26].

Recently, many trigramin-like antiplatelet peptides (or disintegrins) have been reported [2, 4, 13, 17, 27, 31]. Trigramin, an RGD-containing peptide purified from the venom of *Trimeresurus gramineus,* is a specific fibrinogen receptor antagonist with a high binding affinity (Kd, 20 n*M*) for the activated platelet fibrinogen receptor [11, 12]. Those peptides all contain RGD, are rich in cysteine, and bind with a high affinity to integrins on the surface of platelets and other cells. Triflavin, a trigramin-like antiplatelet peptide purified from *Trimeresurus flavoviridis* snake venom is more potent than trigramin [14, 16]. Its

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primary structure consists of seventy amino acid residues, including twelve cysteines with RGD sequence at position 49–51 [15]. Triflavin directly interferes with the interaction of fibrinogen with its specific receptor associated with the glycoprotein IIb/IIIa complex [15, 29]. Our previous studies have demonstrated that triflavin not only inhibits B16-F10 murine melanoma cell-induced lung colonization in C57BL/6 mice in a dose-dependent manner, but also inhibits the adhesion of B16-F10 cells to ECM (i.e., fibronectin, fibrinogen, vitronectin, and collagen type I) [28]. We believe that triflavin exerts its antimetastatic effect primarily through blocking tumor cell adhesion to those ECM. In this study, we further demonstrate that triflavin inhibits tumor cell adhesion by binding to its receptors expressed on B16-F10 cell surface membrane via an RGD-dependent mechanism.

Material and Methods

Materials

T. flavoviridis venom was purchased from LAXOTAN, France, and stored at – 20 °C. Gly-Arg-Gly-Asp-Ser (GRGDS) was purchased from Peninsula Laboratories, USA. GRGES was synthesized by Biochemical Institute, College of Medicine, National Taiwan University. Fluorescein isothiocyanate (FITC) was purchased from Molecular Probe Inc. Triflavin was prepared according to the methods described previously elsewhere [16]. The purified triflavin migrates as a single band and its molecular mass was estimated to be 7,500 daltons on SDS-PAGE (20% gel).

Cell Culture

B16-F10 mouse melanoma cells were obtained from the Institute of Preventive Medicine, Taipei, Taiwan and grown in Dulbecco's minimum essential medium (DMEM) containing 10% FCS and 1% *L*-glutamine. Cells were passaged and harvested for experiments before reaching confluence. Cell culture reagents including FCS were obtained from GIBCO (Grand Island, N.Y., USA).

Adhesion Assays

B16-F10 melanoma cells were detached with EDTA (1 m*M*)/trypsin (0.25%, w/v) and thoroughly washed with DMEM to remove residual FCS. Cells were resuspended in DMEM at a concentration of 1×10^4 cells/ml. Plates (96-well; Costar, USA) were previously coated overnight at 4 °C with 50 µl of triflavin (1.3–13 µ*M*) in phosphate-buffered saline (PBS). A 300-µl aliquot of cells (1×10^4 cells/ml of Hanks' balanced salt solution with glucose and 0.5% bovine serum albumin, BSA) was placed into each well and incubated for 90 min at 37 °C. Nonadherent cells were removed by aspiration and the cells were gently washed with PBS. The adherent cells were then fixed with 2% glutaraldehyde for 10 min and stained with 2% Giemsa for 20 min. Cells were counted with an image analyzer (Pointek Computer, Taiwan), or with 1.0-mm^2 reticle in the eyepiece using a Nikon inverted phase-contrast microscope and viewed at 100 × magnification. For a competition assay, various concentrations of GRGDS and GRGES in a 300-µl aliquot of cells were simultaneously added to triflavin-coated plates. Also, adhesion assays were performed as described above.

Fluorescein-Conjugated Triflavin

Briefly, 1 mg of triflavin was dissolved in 0.2 ml of 0.1 *M* sodium bicarbonate, and then 1 mg of FITC dissolved in 0.1 ml DMSO was freshly prepared immediately before starting the reaction. 20 µl of FITC solution was added to the protein solution. The mixture was then incubated for 1 h at room temperature while continuously being stirred. The reaction was stopped by adding 20 µl of freshly prepared 1.5 *M* hydroxylamine (pH 8.0–8.5) for another 30 min. Finally, the conjugated protein was separated from the residual labeling reagent and hydroxylamine by a Sephadex G-10 column (10 × 300 mm) equilibrated with PBS. The collected fraction was lyophilized, and the protein content was estimated by the Lowry method [23]. Finally, the concentration of FITC-conjugated triflavin was adjusted to 1 mg/ml.

Flow-Cytometric Analysis

B16-F10 cells were detached (using PBS containing 0.5 m*M* EDTA) and thoroughly washed with PBS containing 1% BSA and 2% goat whole serum (GIBCO, USA), and preincubated in the same buffer for 30 min at 4 °C. Cells were directly treated with various concentrations of FITC-triflavin for 60 min at 4 °C, then washed (3 ×) with a buffer solution and finally resuspended to 1 ml/tube for assaying. Fluorescein-labeled cells were assayed with Coulter EPICS dual-laser cytometer (Coulter Electronics). Next, FITC signals were detected and digitized in a log base 10 configuration and the data accumulated on an EPICS computer system. Data were accumulated in a 256-channel resolution and 10,000 cells were counted per experimental group. Fluorescence intensity was directly proportional to the fluorescein label bound on the tumor cell's surface. The relative fluorescence intensity was calculated by subtracting the mean control intensity (i.e., FITC-triflavin in the presence of 5 m*M* EDTA) from the mean experimental fluorescence intensity. All experiments were repeated at least 4 times to ensure reproducibility.

Fluorescence Staining

B16-F10 cells (1×10^5) were placed on four-chamber glass coverslips (Nunc, Naperville, Ill.) and allowed to grow for 2 days. Coverslips with plated cells (50–65% confluent) were washed (3 ×) with PBS to remove serum proteins and fixed in 0.1% paraformaldehyde for 8 min and then washed (2 ×) in PBS. Cells were treated with FITC-triflavin at 37 °C for 1 h. Negative controls were processed in the presence of 5 m*M* EDTA. In addition, representative fields of cells were photographed through an Olympus microscope equipped with epiluminescence optics (VANOX-S, Olympus).

Results

Promotion of Cell Adhesion by Immobilized Triflavin

Our previous study indicated that triflavin inhibits adhesion of B16-F10 cells to ECMs in a dose-dependent manner [28]. Since RGD-containing peptides have been shown to promote cell attachment, we further examined the capacity of the immobilized triflavin to mediate B16-F10 cell adhesion. After 90 min of incubation at a cell

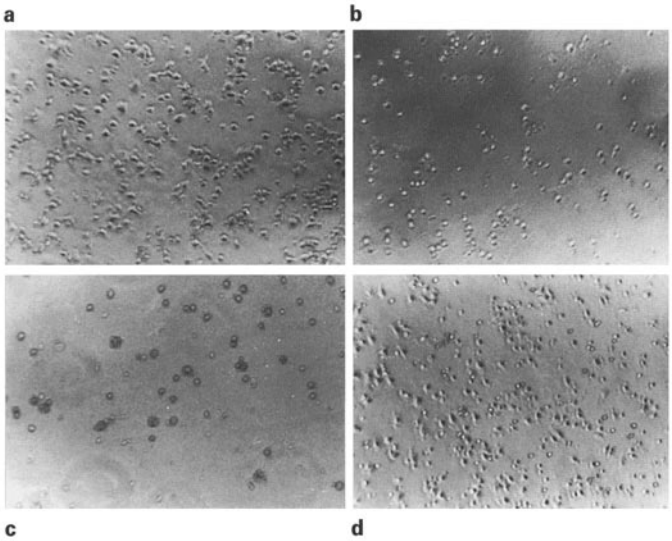


Fig. 1. Adhesion of B16-F10 mouse melanoma cells to immobilized triflavin in the presence of GRGDS or GRGES. Cell adhesion to immobilized triflavin in the absence of GRGDS (**a**; control), or in the presence of GRGDS (1 m*M*; **b**), GRGDS (2 m*M*; **c**) and GRGES (2 m*M*; **d**). Wells were treated with 50 µl of triflavin (5.2 µ*M*) in PBS at 4°C overnight. Cells were harvested with EDTA (1 m*M*)/trypsin (0.25%, w/v) and washed 3 times in serum-free DMEM to remove residual FCS. 300-µl cell aliquot (1 × 10^4/ml) containing GRGDS, GRGES or saline was added to each well. Cells were incubated for 90 min at 37°C. Nonadherent cells were removed by aspiration and the wells washed with PBS. Adherent cells were fixed with 2% glutaraldehyde for 10 min and stained wtih 2% Giemsa for 20 min. × 100.

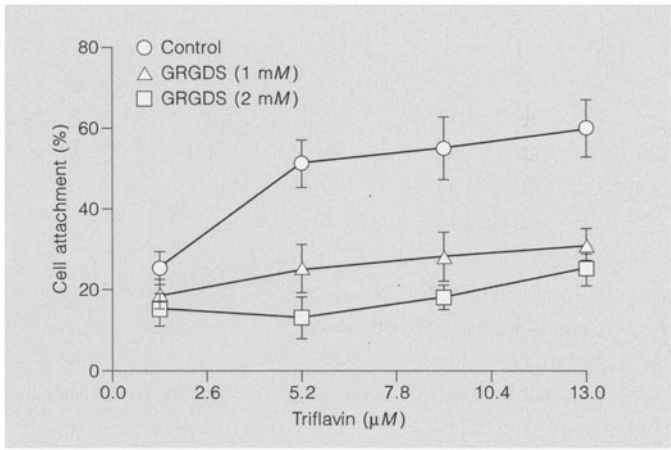


Fig. 2. Effect of GRGDS on B16-F10 cell adhesion to immobilized triflavin. Triflavin was immobilized on wells as described in the legend to figure 1. 300-µl cell aliquot (1 × 10^4 cells/ml) containing 1 m*M*, 2 m*M* GRGDS or saline (control) was added to each well. The cell number added to the wells was taken as 100%. Data are presented as mean ± SEM (n = 4–5).

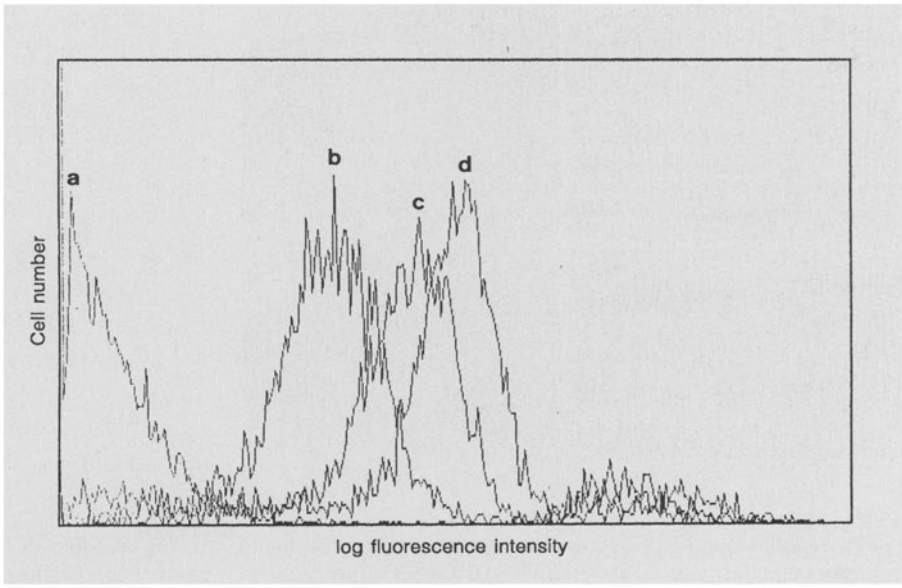
concentration of 1 × 10^4 cells/ml, more than 50% of the cells attached themselves to the immobilized triflavin (fig. 1a). Nevertheless, adhesion of B16-F10 cells to immobilized triflavin was less effective in inducing cell spreading than cells adhering to immobilized fibronectin [28]. As indicated in figure 2, the immobilized triflavin promoted the attachment of B16-F10 cells in a concentration-dependent manner. This finding is in contrast to BSA or gelatin, neither of which promoted cell adhesion at 1 mg/ml (<15%) [data not shown]. Cell adhesion to immobilized triflavin was inhibited by GRGDS in a dose-dependent manner. GRGDS at 1 and 2 m*M* inhibited cell attachment about 50 and 60%, respectively (fig. 1b, c, 2). The control peptide GRGES (2 m*M*) had no significant effect on cell adhesion (fig. 1d).

Effects of GRGDS on FITC-Conjugated Triflavin Binding to B16-F10 Cells

The Arg-Gly-Asp (RGD) sequence is the cell-recognizing domain in a variety of adhesive proteins including fibronectin, fibrinogen, vitronectin and collagen type I [25]. This tripeptide plays an influential role in the interaction of those adhesive proteins with their specific receptors. Our previous investigation indicated that triflavin may bind to RGD recognition sites of the cell surface, thereby inhibiting tumor cell attachment to ECMs [28]. Figure 3 provides further evidence according to which (1) FITC-triflavin directly binds to B16-F10 cells in a concentration-dependent manner and (2) the presence of GRGDS (0.41 and 0.82 m*M*) apparently inhibits triflavin binding to B16-F10 cells (30 and 50%, respectively). This occurrence indicates that the RGD sequence of triflavin plays a prominent role in mediating the binding of triflavin. In the presence of 5 m*M* EDTA, the FITC-triflavin binding was markedly inhihited by 90%, indicating that its binding activity is divalent cation-dependent (fig. 3a).

Immunofluorescence of FITC-Triflavin on B16-F10 Cells

B16-F10 cells may express receptors mediating triflavin binding, as revealed by flow-cytometric analysis (fig. 3). Incubation of FITC-triflavin (0.26 µ*M*) with B16-F10 cells produced weak fluorescence over the surface of the cell (fig. 4a). When FITC-triflavin was added at a higher concentration (1.3 µ*M*), an enhanced fluorescence intensity was observed (fig. 4b). These results closely correspond to the quantitative results obtained by flow-cytometric analysis (fig. 3). However, the fluorescence



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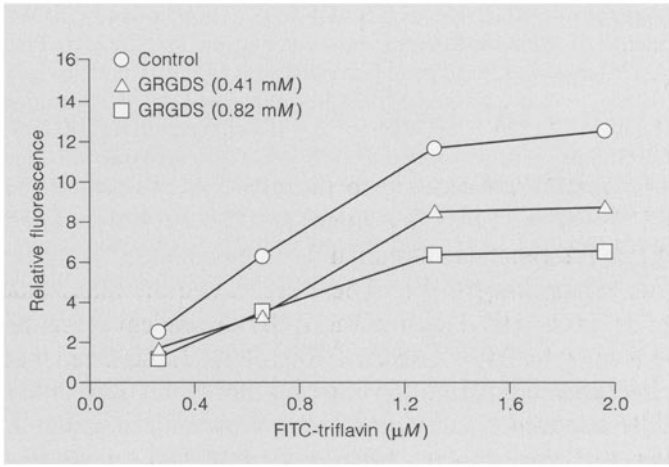

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Fig. 3. Flow-cytometric analysis of FITC-conjugated triflavin binding to B16-F10 cells. Cells were harvested, washed, and resuspended at a concentration of 2×10^5 cells/ml in PBS containing 1% BSA and 2% goat whole serum. FITC-triflavin was added as a probe (see Materials and Methods). **a** The solid lines represent the fluorescence profile of B16-F10 cells. Control (a) is represented by cells incubated in the presence of 5 m*M* EDTA and 0.26 μ*M* FITC-triflavin. On the other hand, cells were incubated with various concentrations of FITC-triflavin [0.26 μ*M* (b), 0.65 μ*M* (c), 1.3 μ*M* (d)] in the absence of EDTA. **b** Binding of isotherm of FITC-triflavin in B16-F10 cells in the absence or presence of GRGDS (0.41 m*M*, 0.82 m*M*). The results are from one representative of four similar experiments.

staining was almost completely inhibited by 1.2 m*M* GRGDS treatment (fig. 4c) and 5 m*M* EDTA treatment (fig. 4d).

Discussion

Many studies have demonstrated that tripeptide RGD is a cell recognition site. RGD appears to be the active binding site within the molecules of triflavin and other disintegrins [6, 15]. Extracellular adhesion-promoting proteins thus likely interact with adhesion receptors of tumor cells via RGD sequence. Dedhar et al. [3] demonstrated that the MG-63 human osteosarcoma cell expresses a receptor complex for collagen type I which recognizes the RGD sequence. In addition, Kieffer et al. [20] reported that the M21 human melanoma cell expresses a vitronectin receptor which mediates cellular adhesion to fibrinogen, von Willebrand factor and vitronectin via an RGD recognition site. Our previous study demonstrated that triflavin inhibited adhesion of B16-F10 cells to ECMs (e.g., fibronectin, fibrinogen, vitronectin and collagen type I) and inhibited B16-F10 cell-induced lung colonization in C57BL/6 mice [28]. Moreover, triflavin inhibited pulmonary tumor metastasis, probably by interfering with cellular adhesive processes. The above results suggest that the RGD sequence within the triflavin molecule is important for receptor recognition. Triflavin inhibits platelet aggregation by directly binding to the glycoprotein IIb/IIIa complex and, thus, competitively interferes

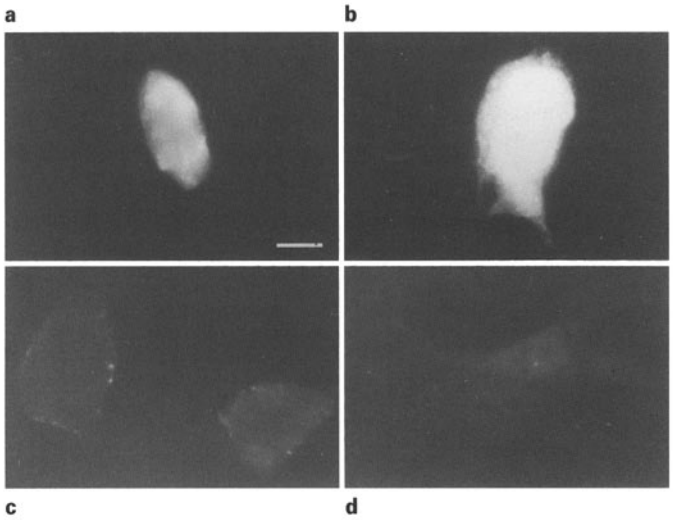

Fig. 4. Fluorescence staining of FITC-triflavin on B16-F10 cells. B16-F10 cells were stained with FITC-triflavin, 0.26 μ*M* (**a**), 1.3 μ*M* alone (**b**), or in the presence of FITC-triflavin (0.26 μ*M*) plus 1.2 m*M* GRGDS (**c**) or in the presence of FITC-triflavin (0.26 μ*M*) plus 5 m*M* EDTA (**d**). All photographs are taken of the same field. Bar: 10 μm.

with fibrinogen binding to the activated platelets. The GPIIb/IIIa complex is a member of structurally related glycoproteins in mammalian cells belonging to the integrin family ($\alpha_{IIb}\beta_3$) [31]. Therefore, it is quite likely that triflavin directly binds to the RGD-dependent integrins expressed by B16-F10 cells. Grossi et al. [7] suggested that carcinoma cells including human cervical carcinoma (MS 751) and human colon carcinoma (clone A) express a plasma membrane receptor (i.e., IR GP IIb/IIIa, IR, immunologically related) that is immunologically and functionally related to the receptor complex of the platelet surface (i.e., GP IIb/IIIa complex). This IR GP IIb/IIIa is a multifunctional receptor that mediates tumor cell adhesion to a variety of biological substrates. Kundsen et al. [22] demonstrated that a heterodimeric glycoprotein complex with a β-subunit biochemically and immunologically similar to platelet IIIa plays a dominant role in the adhesion of human melanoma cells to vitronectin.

On the other hand, B16-F10 mouse melanoma cells have also been shown to express a number of integrins including three different members of a β_1-subfamily that interact with fibronectin, laminin and collagen type I and IV, respectively [24]. However, the expression of the β_3-subunit was restricted exclusively to tumorigenic melanoma cells (i.e., vertical growth phase melanoma and metastastic melanoma) [1]. Since triflavin is known to bind to the RGD-dependent integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$) on platelets, triflavin likely binds to RGD-dependent integrins expressed by B16-F10 melanoma cells [15, 29]. In this study, FITC-triflavin bound to B16-F10 cells in a saturable manner and was susceptible to be blocked by EDTA (5 m*M*), indicating that its binding activity is divalent cation-dependent. Furthermore, GRGDS dose-dependently inhibited both FITC-triflavin binding to B16-F10 cells (fig. 3, 4c) and the cell adhesion to immobilized triflavin (fig. 2), indicating that triflavin binds to B16-F10 cells through an RGD-dependent mechanism. However, residual RGD-independent binding of triflavin might exist (~ 20%, fig. 2), thereby suggesting that triflavin may have recognition sites other than RGD. Another possibility may be due to the insufficient dosage of GRGDS used (2 m*M*). This RGD-independent binding may be due to a subpopulation expression of different cell surface receptors either from different variants of cells or from cells in different stages of cell cycle.

In conclusion, triflavin via its RGD sequence directly binds to its receptors expressed on the surface of B16-F10 melanoma cells, thereby blocking B16-F10 cell adhesion to ECM (i.e., fibronectin and vitronectin). However, the exact identities of the integrins expressed on the B16-F10 cells to which triflavin binds remain unclear and require further study.

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