A role for transferrin receptor in triggering apoptosis when targeted with gambogic acid


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Transferrin receptor (TfR) has been shown to be significantly overexpressed in different types of cancers. We discovered TfR as a target for gambogic acid (GA), used in traditional Chinese medicine and a previously undiscovered link between TfR and the rapid activation of apoptosis. The binding site of GA on TfR is independent of the transferrin binding site, and it appears that GA potentially inhibits TfR internalization. Down-regulation of TfR by RNA interference decreases sensitivity to GA-induced apoptosis, further supporting TfR as the primary GA receptor. In summary, GA binding to TfR induces a unique signal leading to rapid apoptosis of tumor cells. These results suggest that GA may provide an additional approach for targeting the TfR and its use in cancer therapy.

rapid apoptosis | caspases | target identification

Transferrin receptor (CD71, TfR) is a type II transmembrane protein with a molecular mass of 85 kDa as a monomer. TfR interacts with two proteins: transferrin (Tf) and hereditary hemochromatosis protein involved in the cellular transport of iron (1, 2).

TfR expression is increased in dividing cells (3), and its overexpression has been reported in different types of cancers such as glioma, pancreatic, and colon cancers (4, 5). Tf and TfR have been targets for therapeutic intervention in certain cancers. These approaches have primarily consisted of using Tf or Tf to direct a toxin or therapeutic molecule to the cancer cells, the use of antisense- or antibody-mediated therapies directed at TfR or Tf, and iron chelator therapies to starve cells of iron (6, 7). Induction of apoptosis or growth inhibition of tumor cells through these approaches has been reported to take several days (8, 9).

Apoptotic cell death is the consequence of a series of precisely regulated events that are frequently altered in tumor cells. The sequence of events that results in the activation of caspases has been broadly categorized into two pathways, the “extrinsic” pathway (10) and the intrinsic mitochondrial pathway (11, 12). In either case, the ultimate cleavage and activation of the executioner caspases ensures destruction of the cell. Using chemical genetics in our drug discovery program, we discovered gambogic acid (GA) as an apoptosis inducer (13).

We have now discovered TfR as the molecular target for GA, a natural product from the resin of the Gamboge Hanburyi tree. The resin has been used in traditional Chinese medicine (14). GA and its active derivatives bind to the purified TfR independent of the Tf binding site and rapidly activate apoptosis in cells. A short exposure to the drug is sufficient to rapidly activate the apoptosis cascade. The discovery and characterization of the GA/TfR interaction in apoptosis may allow us to develop new ways to exploit TfR and other related proteins for therapeutic purposes.

Methods

Preparation of GA and Derivatives. GA was isolated in overall yield of ∼5% from the easily and widely available gamboge resin. It was purified by converting the crude extract from the gamboge resin into pyridine salt, followed by recrystallization. Derivatives of GA were prepared according to the reported procedure in ref. 13. The characterization of GA derivatives is available as Supporting Methods, which is published as supporting information on the PNAS web site.

Cell Lines and Reagents. Jurkat, T47D, Mes, Mes ADR, and 293T cells were purchased from American Type Tissue Culture (American Type Culture Collection, Manassas, VA). Jurkat/ Bel2, WT mouse embryonic fibroblast (MEF), and Apaf1 null MEFs were provided by D.R.G. Primary human umbilical vein endothelial and human mammary epithelial cells were purchased from Cambrex (East Rutherford, NJ). Detailed growth conditions and reagents are available in Supporting Methods. Anti-TfR antibodies and holo-transferrin were used as described in Supporting Methods.

Apoptosis Assays. DAPI staining, propidium iodide viability, and cell cycle analysis were done by using standard methods explained briefly in Supporting Methods. Caspase induction assay was done as described in ref. 15. Cell proliferation assay: The CellTiter 96 AO〈sub>450</sub><sup>›</sup> Assay (Promega) was used to determine the 50% growth inhibition (GI<sub>50</sub>) values for the compounds according to the manufacturer’s instructions.

Identification of GA Target. Membrane proteins were solubilized, run on SDS/PAGE, stained, and followed by in-gel tryptic digest according to standard procedures (Supporting Methods). Protein identification was performed by LC/MS/MS (Centre Hospitalier de l’Universite Laval Research Centre, Quebec), and the actual peptides identified are listed in Supporting Methods.

Immunofluorescence, Immunohistochemistry, and Electron Microscopy. For detailed immunofluorescence, immunohistochemistry, and electron microscopy see Supporting Methods. T47D cells were pretreated with 2 μM GA for 15 min at 37°C washed with PBS, and then labeled for 30 min with 1 μg/ml FITC-labeled mouse anti-human TfR (RDI, Flanders, NJ) at 37°C and mounted. For electron microscopy, sections were examined at an accelerating voltage of 60kV by using a Zeiss EM10C electron microscope core facility (Veterans Hospital, San Diego). More details and methods used for the immunoprecipitations and Western blotting are described in Supporting Methods.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: GA, gambogic acid; biotin-GA, biotin-conjugated GA; fluorescein-GA, fluorescein-conjugated GA; LC/MS/MS, liquid chromatography tandem mass spectrometry; MEF, mouse embryonic fibroblast; siRNA, short interfering RNA; Tf, transferrin; TfR, transferrin receptor.

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Short Interfering (siRNA) Transfections, cDNA Synthesis, and Real-Time PCR. Chemically synthesized human transferrin receptor and caspase-8 siRNA oligos were used (Ambion, Austin, TX). The target sequence for TIR siRNA was 5’ AAC TCT TGG GTG GCC AGC 3’ and for caspase-8 siRNA was 5’ AAG GAA AGT TGG ACA TCC TGA 3’. The control siRNA oligos, human cyclophilin, and negative control scrambled siRNAs were also from Ambion (16).

Standard procedures were used for cDNA synthesis and quantitative PCR experiments (see Supporting Methods) cDNA was made by using the Retroscript cDNA synthesis kit (Ambion) according to the manufacturer’s instructions. Quantitative PCR was done by Sybrgreen incorporation with the Roche Molecular Biochemicals, Mannheim, Germany by using standard conditions. Data were normalized against the housekeeping gene, cyclophilin. The cells transfected with cyclophilin as a control were normalized against glyceraldehyde phosphate dehydrogenase.

Binding Assays. Jurkat cells were incubated with tritium-GA at 1 μM at 37°C with or without 20 μM unlabeled GA. At indicated time points, bound tritium-GA was quantitated by liquid scintillation counting. TIR-coated wells were incubated with GA-biotin as described above, washed, and incubated with nontagged analogs or binding/washing buffer as a control. Wells were incubated with Europium-Streptavidin, then quantified after incubation with Enhancement Solution (PerkinElmer) by measuring time-delayed fluorescence on a Wallac Victor plate reader (PerkinElmer) according to manufacturer’s instructions.

Results

Gambogic Acid Activates Apoptosis and Inhibits Tumor Growth in Vitro. GA triggers morphological changes in cells typical of apoptosis. Membrane blebbing is induced within 15 min in Jurkat cells as observed by electron microscopy (Fig. 1A). To verify that caspases are critical in this pathway, we tested the effect of a pan-caspase inhibitor, MX-1013, on apoptosis (17). We observed that morphological changes (Fig. 7, which is published as supporting information on the PNAS web site), proteolytic cleavage of caspases, and apoptosis (data not shown) depend on activation of caspases.

GA derivatives were made to elucidate the structure activity relationship (13). The tricyclic ring and the α, β unsaturated ketone, as indicated, are essential for the activity of GA (Fig. 1B). The saturated ketone derivative (inactive GA) does not activate caspases in cells. GA activates caspases in our cell-based assay (15) and inhibits cell growth in various tumor cell lines at submicromolar potencies; this effect is also seen in multidrug resistant human uterine sarcoma cell line (18) as shown in Table 1 where standard chemotherapeutics showed a 100- to 1,000-fold decrease in potency (data not shown).

Table 1. GA inhibits growth in various tumor cell lines

<table>
<thead>
<tr>
<th>Cell</th>
<th>Tissue</th>
<th>GI50, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D</td>
<td>Breast</td>
<td>630</td>
</tr>
<tr>
<td>ZR751</td>
<td>Breast</td>
<td>400</td>
</tr>
<tr>
<td>HL60</td>
<td>Lymphocyte</td>
<td>115</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Lymphocyte</td>
<td>168</td>
</tr>
<tr>
<td>Calu1</td>
<td>Nonsmall cell lung</td>
<td>550</td>
</tr>
<tr>
<td>MES</td>
<td>Uterine</td>
<td>300</td>
</tr>
<tr>
<td>MES ADR*</td>
<td>Uterine</td>
<td>1,000</td>
</tr>
</tbody>
</table>

*Multidrug resistance pgp overexpressed (19).
bound protein, we also identified TfR peptides. We further confirmed the identification of TfR by Western blotting in immunoprecipitations by using fluorescein-GA (Fig. 2D).

**GA Binds to TfR and Does Not Compete with Tf.** To evaluate and confirm the binding of GA to TfR, we performed a series of experiments by using purified TfR containing the extracellular domain in a binding assay with biotin-GA. We determined that biotin-GA binds to TfR with a specificity of binding. To further understand the GA/TfR interaction, we showed that biotin-GA bound to TfR can be displaced by active GA derivatives. In competition experiments, biotin-GA at 1 μM was premixed with increasing amounts of GA or the inactive-GA as a competitor. Amounts of bound Eu-Streptavidin were quantified by measuring time-delayed fluorescence. (C) GA bound to TfR in vitro can be displaced by active GA derivatives. TfR-coated wells were incubated with biotin-GA as described, washed, and incubated with GA or binding/washing buffer as a wash off control. (D) Binding of biotin-GA and tritium-GA to TfR is not inhibited by either apotransferrin or holo-transferrin. Binding of biotin-GA and tritium-GA to Jurkat cells (hatched) or in vitro Tf-binding (solid) in the presence of 1 μM GA, 50 μg/ml apotransferrin, or 50 μg/ml holo-transferrin is shown in this graph.

**Table 2. Functional activities of GA derivatives correlate with their binding to TfR**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Competition IC50 (μM)</th>
<th>Activity in apoptosis induction assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fold caspase-3 activation</td>
</tr>
<tr>
<td>GA</td>
<td>4.1</td>
<td>18</td>
</tr>
<tr>
<td>Methyl-GA</td>
<td>3.6</td>
<td>24</td>
</tr>
<tr>
<td>Inactive-GA</td>
<td>&gt;30</td>
<td>1.2</td>
</tr>
<tr>
<td>α,β unsaturated backbone</td>
<td>12.7</td>
<td>10</td>
</tr>
<tr>
<td>α,β saturated backbone</td>
<td>&gt;30</td>
<td>1</td>
</tr>
</tbody>
</table>

ND, not determined.
TfR expression. Jurkat cells were treated with holo-transferrin alone (50 μM) or 50 μM desferrioxamine (DFO) or 50 μM ferric nitrate [Fe3(NO)3] for 1 h and treated as described in A. (C) GA interferes with TfR receptor internalization. T47D cells were treated with DMSO or 2 μM GA for 15 min and further treated with FITC-conjugated anti-TfR for 30 min at 37°C. The cells were then fixed with methanol at −20°C for 5 min, washed with PBS, and mounted with Vectashield mounting medium. Representative of three independently confirmed experiments. (D) GA interferes with TfR internalization as indicated by cell surface TfR expression. Jurkat cells were treated with holo-transferrin alone (50 μg/ml) for 3 or 5 min, GA alone (5 μM) for 2 min, or pretreated for 5 min with GA followed by 1, 3, 5, or 10 min of holo-transferrin treatment. Cells were then stained with FITC-conjugated anti-transferrin receptor antibody for 30 min at 4°C. After washing, cells were analyzed on a Becton Dickinson FACs Calibur. Data are shown as mean fluorescence units. Results were confirmed in three independent experiments.

**Fig. 4.** Known mechanisms of iron regulation do not overlap with GA-mediated apoptosis. (A) The binding of holo-Tf to TfR has no effect on GA-induced apoptosis. Jurkat cells were treated with holo-transferrin for 30 min and subsequently treated with DMSO or 1, 2.5, or 5 μM GA for 4 h, after which cell viability was measured as described. (B) GA-induced apoptosis through TfR is not iron-dependent. Jurkat cells were treated with 10 μM desferrioxamine (DFO) or 50 μM ferric nitrate [Fe3(NO)3] for 1 h and treated as described in A. (C) GA interferes with TfR receptor internalization. T47D cells were treated with DMSO or 2 μM GA for 15 min and further treated with FITC-conjugated anti-TfR for 30 min at 37°C. The cells were then fixed with methanol at −20°C for 5 min, washed with PBS, and mounted with Vectashield mounting medium. Representative of three independently confirmed experiments. (D) GA interferes with TfR internalization as indicated by cell surface TfR expression. Jurkat cells were treated with holo-transferrin alone (50 μg/ml) for 3 or 5 min, GA alone (5 μM) for 2 min, or pretreated for 5 min with GA followed by 1, 3, 5, or 10 min of holo-transferrin treatment. Cells were then stained with FITC-conjugated anti-transferrin receptor antibody for 30 min at 4°C. After washing, cells were analyzed on a Becton Dickinson FACs Calibur. Data are shown as mean fluorescence units. Results were confirmed in three independent experiments.

**GA Potentially Interferes with TfR Internalization and Is Independent of Iron Regulation.** Because TfR is important for cellular iron regulation and interfering with this pathway in various ways can lead to growth inhibition in cells, we investigated the effects of iron and transferrin-dependent iron regulatory mechanisms during GA-mediated apoptosis. First, we examined whether binding of Tf to the receptor has any effect on GA-induced apoptosis. Even in the presence of saturating levels of holo-Tf, GA was capable of inducing apoptosis in Jurkat cells (Fig. 4A).

We also determined whether GA-mediated apoptosis was iron-dependent or sensitive to iron deprivation. We used a clinically tested iron-chelator, desferrioxamine (22) ferric nitrate (Fig. 4B) and ferrous citrate (Fig. 8C) to get a quantitative idea about the cell surface localization of TfR. Although holo-Tf lead to a reduced TfR staining, this effect may be due to interference in their binding sites as indicated by the same experiment repeated at 4°C (Fig. 8C). GA treatment alone for 2 min caused increased cell surface TfR levels. Similar data were also obtained by monitoring for Tf endocytosis by using alexa-fluor-labeled transferrin (Fig. 8B). We observed an increased TfR expression on cell surface on GA treatment. These results suggested that binding of GA to TfR and the subsequent induction of apoptosis is unrelated to Tf binding and iron transport.

**Down-Regulation of TfR in Cells Affects Sensitivity to GA-Induced Apoptosis.** To understand the correlation of TfR levels and susceptibility to GA-mediated apoptosis and to determine the therapeutic advantage of GA, we evaluated its effect on tumor cells that overexpress TfR on their cell surface (T47D and 293T) and primary normal human mammary epithelial and human umbilical vein endothelial cells. We observed that tumor cells treated for only 2 h with GA underwent significant apoptosis compared with that observed for primary normal cells (Fig. 5A). There was no correlation between the proliferation rates and sensitivity to GA (data not shown); however, we observed a correlation between the level of TfR expression and rapidity of GA-induced apoptosis (Fig. 5A). Although, 293T cells and T47D cells have a comparable level of apoptosis when treated with 1 μM GA, the latter cells are more sensitive to apoptosis even at lower concentrations of GA (data not shown). To further validate that GA is the target for GA, we used RNA interference assays with siRNA duplexes to inhibit the expression of TfR protein. Studies were performed to evaluate sensitivity of the transfected cells toward GA. Down-regulation of endogenous TfR after transfection with the siRNA oligonucleotide was confirmed by mRNA analysis (Fig. 5B). Western blot analysis (Fig. 5C), and determination of cell surface receptor expression (data not shown). TfR-specific siRNA transfections showed ∼70% inhibition of TfR expression after 48 h. Down-regulation of TfR by siRNA was specific, as shown by analysis of other cell cycle (24) and apoptosis genes such as p21, cdk2, caspase-8, and clathrin (Fig. 9A, which is published as supporting information on the PNAS web site). After down-regulation of endogenous TfR by using siRNA, 293T cells displayed a significant reduction in apoptosis induced by GA (Fig. 5D). Similar results were obtained by using T47D breast cancer cells (data not shown). The doubling times in the various transfected cells were not significantly altered (Fig. 9B). We also used paclitaxel as a known cytotoxic agent and showed that the down-regulation of TfR did not result in a reduction in apoptosis due to paclitaxel (Fig. 9B), thus indicating that TfR down-regulated cells are not resistant to all apoptotic stimuli.
TfR Signaling Mechanism for Apoptosis Induction. GA induces apoptosis through a very robust engagement of the apoptotic pathway. These data include activation of caspase-8, cleavage of Bid, the release of cytochrome c from the mitochondria and activation of caspase-3 (Fig. 6A). Because cleavage of caspase-8 may not empirically imply its enzymatic activation (25), a biotinylated caspase inhibitor was used that binds to the active site of caspasas, including caspase-8 and characterized GA-mediated caspase activation. It was observed that GA activates caspase-8 similar to that reported for anti-Fas activation (Fig. 10A, which is published as supporting information on the PNAS web site). However, interfering with the death receptor signaling by using Fas-Fc or TNFR-Fc (data not shown) chimeric proteins did not have any effect on GA-mediated cell death.

To better define the relative involvement of the extrinsic and intrinsic pathways in GA-mediated apoptosis, a genetic approach was used. Down-regulation of caspase-8 in cells by RNA interference (Fig. 10B) decreased the sensitivity to GA-induced apoptosis. Although Jurkat cells are of type II apoptotic phenotype, recent reports (29) on TRAIL-induced apoptosis in these cells warrants further clarification on this topic. Although, caspase-8 activation seems to be required in GA-mediated signaling, the involvement of other initiator caspsases, i.e., caspase-10 and caspase-2, cannot be ruled out, especially because of their importance in certain apoptotic pathways (30, 31).

TfR and Tf have been previously identified as targets for cancer therapy. Existing antibody-based approaches may have restricted effectiveness due to inadequate drug delivery and/or immunogenicity issues when using antibody fusion proteins. Iron chelator therapies appear to have clinical activity and are under investigation and in early clinical trials (32). However, iron chelators are reported to act on multiple targets, including those involved in cell cycle progression, which may or may not enhance efficacy or affect toxicity of these compounds. Although TfR has been a target for cancer treatment, the approach reported herein
appears to result in a previously unknown mechanism for TIR in the induction of apoptosis.

The specificity of GA/TIR interaction is also demonstrated in activating apoptosis because close analogs of GA that do not compete for receptor binding also do not activate caspases in cells. A GA derivative has also demonstrated significant antitumor efficacy in rodent tumor models with little toxicity (unpublished data). We demonstrate a correlation in TIR expression levels between tumor and normal cells and the sensitivity to GA, which may explain, at least in part, the selectivity seen in vivo. A more detailed evaluation of the pharmacokinetics and biodistribution of the drug and the target expression profile will help to better understand the therapeutic advantage observed by this class of molecules.

These results report the identification and characterization of a molecule (GA) that specifically targets TIR engaging a previously unreported mechanism of action to induce apoptosis. It appears from this body of work that GA interferes with TIR internalization leading to the initial, and rapid, signal for apoptosis. We also demonstrate that GA and TIR bind to independent sites on the receptor, and it appears that GA is not competed by TIR. These studies also suggest a requirement of GA for TIR-mediated rapid apoptosis because the mere down-regulation of TIR does not activate this pathway. Whether GA causes additional conformational changes in the receptor, thereby recruiting the death machinery in an unprecedented manner, remains to be uncovered.

With our continuing efforts, we suggest that the GA/TIR discovery may lead to a new generation of anti-cancer drugs and targeting mechanisms that will be synergistic with existing treatments. Identifying roles for other adaptor proteins or signaling molecules in this GA-induced TIR pathway will possibly identify additional therapeutic targets.

We acknowledge the technical assistance provided by Candace Crogan-Grundy. This work is supported in part by National Institutes of Health Grant 5R44CA091811-03 (to S.K.).