Identification of a Natural Compound by Cell-Based Screening That Enhances Interferon Regulatory Factor-1 Activity and Causes Tumor Suppression

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Abstract

The transcription factor interferon regulatory factor-1 (IRF-1) is induced by many tumor-suppressive stimuli and can mediate antiproliferative and proapoptotic effects in cancer cells. Thus, identifying agents that enhance IRF-1 activity may be an effective approach to cancer therapy. A cell-based screening assay was developed to identify extracts and compounds that could enhance IRF-1 activity, using an IRF-1–dependent luciferase reporter cell line. Through this approach, we identified a natural product extract and a known active component of this extract, baicalein, which causes a marked increase in IRF-1–dependent reporter gene expression and IRF-1 protein, with modulation of known IRF-1 targets PUMA and cyclin D1. Baicalein causes suppression of growth in vitro in multiple cancer cell lines in the low micromolar range. IRF-1 plays a role in this growth suppression as shown by significant resistance to growth suppression in a breast cancer cell line stably transfected with short hairpin RNA against IRF-1. Finally, intraperitoneal administration of baicalein by repeated injection causes inhibition of growth in both xenogeneic and syngeneic mouse models of cancer without toxicity to the animals. These findings indicate that identifying enhancers of IRF-1 activity may have utility in anticancer therapies and that cell-based screening for activation of transcription factors can be a useful approach for drug discovery.

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Introduction

The interferon (IFN) regulatory factors (IRF) play a vital role in a variety of biological processes including growth regulation and immune activation (1). IRF-1 was the first member of the IRF family of transcription factors to be identified and was originally described as a transcription factor induced by IFNs that upregulates transcription of IFN-inducible genes. However, IRF-1 can also be effectively induced in most cell types after exposure to many other stimuli including retinoic acid (2), ionizing radiation (3), and other cytokines (1). Interestingly, another IRF protein, IRF-2, binds the same sequence as IRF-1 but downregulates or blocks upregulation of the genes inducible by IRF-1. While IRF-1 has been found to act as a tumor suppressor, IRF-2 was determined to be pro-oncogenic (4).

The role of IRF-1 as a tumor suppressor is well established. IRF-1−/− embryonic fibroblasts can become transformed by introduction of just one oncogene, whereas wild-type embryonic fibroblasts require at least 2 oncogenes for transformation (5). NIH3T3 cells transformed by IRF-2 overexpression can be reverted to the nontransformed phenotype by overexpression of IRF-1 (4). Furthermore, NIH3T3 cells transformed by the oncogenes c-myc and fosB can be reverted to their normal phenotype by ectopic expression of IRF-1 (6). This is remarkable, as IRF-1 could suppress these unrelated oncogenes as opposed to replacing the same deficient tumor suppressor. This observation has been confirmed in vitro and in vivo using a conditionally activated IRF-1 system in transformed NIH3T3 cells (7). Using microarray analysis of inducibly transformed NIH3T3 cells in addition to inducible IRF-1 activity, cyclin D1 was found to be a key downregulated element in the tumor suppression seen with IRF-1 expression (8).

IRF-1 has proven to be a mediator of apoptosis for novel and established agents against cancer. For example, IRF-1 has been found to mediate apoptosis of cancer cells through upregulation of TNF-related apoptosis-inducing
ligand (TRAIL) by IRF-1 in retinoic- and IFN-induced apoptosis (9). Fulvestrant, an antiestrogen that has successfully completed clinical trials, causes apoptosis in susceptible breast cancer cells, for which dominant-negative IRF-1 cells were found to be resistant to this apoptosis (10). Also, IRF-1 has been determined to mediate the apoptotic effects of tamoxifen in ER-poor, acutely damaged, human mammary epithelial cells (11). Although technically not breast cancer, these human mammary epithelial cells have been acutely damaged by HPV-E6, which inactivates p53 and is considered to be an oncprotein.

In addition to inhibiting proliferation and survival of cancer cells, IRF-1 enhances the immunogenicity of tumor cells in part through enhancing IRF-1–dependent expression of MHC proteins. Previously, we showed increased expression of MHC class I and II proteins in cancer cells transfected with an IRF-1 expression vector and found that these cells became immunogenic (12). This was confirmed in another study using an estradiol-regulated inducible IRF-1 system in a hepatic cancer cell line (13).

More recently, we showed that overexpression of IRF-1 using Ad-IRF-1, a recombinant adenovirus expressing IRF-1, induced apoptosis of cancer cells in vitro and in vivo (14–16). IRF-1 expression resulted in apoptosis in mouse breast cancer cell lines in vitro and tumor growth suppression in vivo; this seemed to be caspase-mediated (14). Ad-IRF-1 caused apoptosis in human breast cancer cell lines, suppressed tumor growth in a xenogeneic model of breast cancer, and showed upregulation of p21 and downregulation of survivin (15). We further showed that apoptosis involves both the extrinsic (death receptor) and intrinsic (mitochondrial) apoptotic pathways, but no death ligand seems to be involved (16).

In the intrinsic pathway, we showed that IRF-1 transcriptionally upregulates the mitochondrial proapoptotic protein PUMA in a p53-independent fashion (17).

Given the studies showing the tumor-suppressive activity of IRF-1, an attractive therapeutic strategy may be to identify enhancers of IRF-1 activity based on screening for extracts and compounds that can enhance IRF-1–dependent gene expression. This strategy has been studied previously for the well-known transcription factor, STAT-1, which is the classical upstream regulator of IRF-1 expression (18). However, STAT-1 seems to have protumorigenic activities in certain contexts (19), which may be avoided by enhancement of a more downstream tumor-suppressive factor. Therefore, we developed a cell-based screening system using a luciferase reporter under the control of an IRF-1–dependent promoter sequence. We have successfully identified a natural compound, baicalein, capable of increasing IRF-1 activity that may have therapeutic or chemopreventive potential in humans. Baicalein is a flavonoid that is thought to be one of the active components of the traditional Asian medicine (TAM) extract Huang Qin, which comes from the root of Scutellaria baicalensis (SB). Baicalein has numerous purported activities, but we are the first to link it to IRF-1 activity. Baicalein causes tumor suppression of cancer cells in vitro and in vivo, further suggesting that identification of modulators of transcription factor function may be a useful strategy in the treatment of cancer.

Materials and Methods

Cell lines and culture

The human cancer cell lines AGS, MDA468, BT549, and SKBR3 were from American Type Culture Collection (ATCC) and passaged for less than 6 months after resuscitation. The murine carcinoma cell line C3L5 was described previously (14) and was tested for murine pathogens and mycoplasma and found to be negative. Cell line cross-contamination is unlikely, as these murine cells formed tumors in syngeneic immunocompetent mice. Generation of clone 23 of IRF-1–shRNA/MDA468 (MDA468-C23) is described in Supplementary Methods. All cell lines were cultured as described in Supplementary Methods.

Construction of recombinant adenosviruses and transfection

Ad-IRF-3, which expresses the constitutively active form of IRF-3, was obtained from M.A. Rivieccio and previously described (20). Ad-null (empty vector) and Ad-IRF-1 are previously described (14).

Construction and transfection of IRF-1–luciferase reporter plasmid: selection, characterization, and screening of transfected cells

To construct a cell-based system with which IRF-1–dependent gene activation could be quantified, we used the pGL4.20 luciferase reporter vector from Promega, which contains the firefly luciferase gene. The IRF-1–dependent promoter sequence, which contains 3 tandem repeats of the IRF-1 binding sequence and a minimal promoter sequence, 5′-CAGTACTCTACTTTCATAG-TACTTTCAGTTTCAATTTTCA GTTCATAGACACTAGGGGTATATAATGGAAGCTCGACTTCCA-GCTTG-3′, was cloned into the reporter vector pGL4.20. MDA468 and AGS cells were transfected with this plasmid using SuperFect (Qiagen). Cells were selected against 0.5 μg/mL puromycin, and stable colonies were picked and expanded. For characterization, cells were seeded at 10,000 cells/well in 96-well plates and incubated overnight. Clones were then assayed for low basal activity.
luciferase activity and prominent induction following treatment with Ad-IRF-1 and retinoic acid. Assessment of luciferase activity is described in Supplementary Methods. The best clones were expanded and used for subsequent experiments as the reporter cell lines IRF-1–luc/AGS and IRF-1–luc/MDA468. For screening for enhancers of IRF-1 activity, cells were seeded and 1 μL of 40 natural product extracts (Chinese University of Hong Kong, China) was added to each well in duplicate to a final concentration of 20 μg/mL by robotic transfer with appropriate controls; or SB, active components, or appropriate controls were added as indicated and luciferase activity assessed at 30 hours. Transient luciferase reporter assay using IRF-1 luciferase reporter and β-gal plasmid is described in Supplementary Methods.

**Immunoblotting**

Cells treated with SB extract, baicalein, baicalin, or wogonin as indicated were lysed, cleared by centrifugation, and immunoblotted as previously described (14–17).

**Cell proliferation assay**

Cell proliferation was measured by MTT assay as previously described. MTT conversion to formazan dye correlates with the number of living cells (14–17).

**Animals and in vivo tumor growth suppression**

Six-week-old female SCID-Bg mice (Charles River) had AGS tumor cells (5 × 10^6 cells per animal) implanted subcutaneously with Matrigel in the flank. Mice were ranked by tumor volume and randomized into groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ad-IRF-1 (MOI)</th>
<th>RA (μmol/L)</th>
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<tr>
<td>No treatment</td>
<td>NI</td>
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<tr>
<td>Empty vector Ad</td>
<td>Ad-null (MOI 50)</td>
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<td>Ad-IRF-1 (MOI 5)</td>
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<td>Ad-IRF-1 (MOI 25)</td>
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Figure 1. Characterization of reporter cell lines and screening for enhancers of IRF-1 transcriptional activity. A, IRF-1−luc/AGS and IRF-1−luc/MDA468 cells were plated and transfected with Ad-IRF-1. After 24 hours, luciferase activity normalized to cell viability was determined as described in Materials and Methods. Error bars are SD. B, IRF-1−luc/AGS cells were plated and treated with no infection (NI), adenovirus, or retinoic acid for 24 hours, after which luciferase activities were quantitated. *, P = 0.01; **, P = 0.003. C, IRF-1−luc/AGS cells were plated and treated with 1 μL natural extract per well, and after 30 hours, luciferase activity normalized to cell viability was determined as described in Materials and Methods. Positive controls: Ad-IRF-1, RA, retinoic acid; MOI, multiplicity of infection.
with equal number of sizes. Treatment was initiated on
day 7, when tumors were approximately 70 mm³; with
20 mg/kg of baicalein, genistein, or carrier adminis-
tered by intraperitoneal injection 5 times per week.
Tumor size was assessed by perpendicular caliper mea-
surements conducted weekly. For C3L5, 6-week-old
female C3H/HeJ mice (Jackson Laboratory) were
implanted with 5 x 10⁵ C3L5 cells per animal in the
mammary fat pad and ranked and randomized as ear-
lier. Baicalein or carrier control treatment was initiated
at 20 mg/kg on day 5, administered by intraperitoneal
injection 5 times per week. Tumor size was assessed by
perpendicular caliper measurements every 2 to 3 days.
Tumor volumes were calculated using the formula
\( \pi/6 \times (\text{larger diameter}) \times (\text{smaller diameter})^2 \), \( n =
5–7 \) per group. Experimental protocols were approved
by the Institutional Animal Care and Use Committee at
City of Hope.

Statistical analyses
Experiments were carried out in triplicate or more,
with \textit{in vitro} data presented as mean ± SD and \textit{in vivo}
data presented as mean ± SEM. Statistical comparison of
values were made using 2-tailed Student’s \( t \) test, and
statistical significance was considered to be present when
\( P < 0.05 \).

Results
Characterization of reporter cell lines
We first validated the sensitivity and specificity of the
reporter cell lines. IRF-1–luc/AGS and IRF-1–luc/
MDA468 were transfected with different multiplicity of
infection of Ad-IRF-1, and luciferase activity was deter-
mined after 24 hours. As shown in Figure 1A, luciferase
activity was dramatically increased after Ad-IRF-1 trans-
fection in a dose-dependent manner in both clones,
validating the plasmid reporter construct. Because we
were interested in compounds that enhance IRF-1 activity
specifically, we chose IRF-1–luc/AGS for further charac-
terization and screening because AGS cells do not
express STAT-1 (17), which is a known upstream regulator
of IRF-1. To further confirm the specificity of the assay,
treatment of IRF-1–luc/AGS cells with recombinant
Ad-IRF-3, which results in expression of a constitutively
activated IRF-3 (20), caused no significant change in
luciferase activity (Fig. 1B). To further test the sensitivity
of the assay for cell-permeable molecules, we treated IRF-
1–luc/AGS with retinoic acid, which upregulates IRF-1 by
a non-STAT-1–mediated mechanism (2). Because IFN-\( \gamma \)
upregulates IRF-1 through STAT-1, this cytokine could
not be used either for characterization or as a control and is
also not comparable with the cell-permeable small mole-
cules to be screened. Statistically significant differences in
luciferase reporter activity were seen with retinoic acid at
1 \( \mu \)mol/L (\( P = 0.01 \)) and at 10 \( \mu \)mol/L, with approximately
2-fold induction (Fig. 1B; \( P = 0.003 \)). These assays were
highly reproducible and showed little interwell variabil-
ity. Thus, the IRF-1–luc/AGS cell line seems to be a
sensitive and specific reporter for the activity of IRF-1.

Screening for enhancers of IRF-1 transcriptional
activity
Natural extracts have been long recognized as a
potential source of anticancer drugs. Given the impor-
tance of IRF-1 as a tumor suppressor, the isolation of
natural product extracts that enhance the activity of this
protein would represent a novel approach to cancer
therapy and possibly chemoprevention. To identify such
extracts, IRF-1–luc/AGS cells were seeded in 96-well
plates and then treated with 40 TAM natural extracts
at a final concentration of 20 \( \mu \)g/mL. After 30 hours,
luciferase activity was measured and normalized to cell
viability. From this screen, we identified 4 herbal extracts (4, 22, 28, and 35; Fig. 1C) that increased

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**Figure 2.** SB enhances IRF-1 activity. A, IRF-1–luc/AGS cells were treated
with indicated concentrations of SB extract. Luciferase activity was
determined after 30 hours as described in Materials and Methods.
B, IRF-1–luc/MDA468 cells were treated with SB extract for 30 hours,
after which luciferase activity was measured. C, AGS cells were treated with SB
extract for 24 hours or with 20 \( \mu \)g/mL SB extract for the times indicated.
Cell lysates were immunooblotted for IRF-1, p21, and \( \beta \)-actin.
Densitometric ratios of indicated protein over \( \beta \)-actin and then normalized
to 0 treatment are listed below each band as appropriate.
IRF-1–dependent luciferase activity by significantly more than 3-fold. Among these, the ethanol extract from C4, SB was the greatest enhancer of IRF-1–dependent luciferase activity (Fig. 1C).

**SB enhances IRF-1 activity**

To further investigate and confirm the effect of SB on IRF-1 activity, IRF-1–luc/AGS cells were treated with SB extract and normalized luminescence was measured 30 hours later. As shown in Figure 2A, SB increased luciferase activity in IRF-1–luc/AGS cells in a dose-dependent manner. Luciferase activity was significantly increased at a dose of 1 μg/mL of SB (P < 0.0001). At 20 μg/mL, SB showed maximal effects, enhancing IRF-1–dependent luciferase activity by more than 3-fold.

To determine whether SB enhances IRF-1 activity in other cell types, we also treated IRF-1–luc/MDA468 cells with SB. As shown in Figure 2B, in these cells, SB also induced a significantly increased activity at 1 μg/mL (P = 0.001) and reached approximately 3-fold induction in normalized luciferase activity at 5 μg/mL. This confirms that the SB activity on cancer cells can be generalizable to other cancer cell types including breast cancer cells.

![Figure 3](https://mct.aacrjournals.org/doi/fig/10.1158/1535-7163.MCT-11-0304)

**Figure 3.** Baicalein enhances IRF-1 activity and upregulates IRF-1 expression. A, evaluation of active components of SB in IRF-1–luc cells. Top, structures of baicalein, baicalin, and wogonin are shown. IRF-1–luc/AGS cells were plated, treated with indicated component, and assayed at 30 hours as described in Materials and Methods. *, P = 0.0063 versus control. Bottom, IRF-1/MDA-468 cells were plated, treated, and assayed as in Materials and Methods. *, P = 0.0004 versus control. B, AGS cells were treated with the indicated concentrations of baicalein, baicalin, and wogonin for 24 hours. Cell extracts were assessed for IRF-1 and β-actin by immunoblotting. Densitometric ratios of indicated protein over β-actin and then normalized to 0 treatment are listed below each band as appropriate for B–D. C, immunoblot showing dose dependence and time course of IRF-1 and PUMA protein expression, as well as cyclin D1 suppression, in AGS cells treated with baicalein. AGS cells were either left untreated (C) or treated with baicalein at the indicated doses. Cells were collected after 24 hours. For time course, cells were treated at 20 μmol/L and collected at the times indicated. D, p53 mutant cell line BT549 was treated with baicalein, and immunoblotting of lysates was carried out after 24 hours as indicated.
To further confirm whether SB enhances IRF-1 activity by upregulating IRF-1, we examined IRF-1 protein levels in AGS cells by immunoblotting in a time- and dose-dependent manner. AGS cells were treated with SB extract for 24 hours and were clearly found to have increased protein levels at 10 and 20 μmol/mL at 24 hours in a dose-responsive fashion (Fig. 2C). Moreover, AGS cells incubated with 20 μg/mL SB extract showed increased IRF-1 protein at 24 hours posttreatment, which further increased at 48 and 72 hours (Fig. 2C). We also carried out immunoblotting for p21, which is known to be increased by IRF-1 expression (15), and found that p21 increases in concert with IRF-1 (Fig. 2C).

**Baicalein enhances IRF-1 activity and upregulates IRF-1 expression**

The active components of SB are purported to be flavones, including baicalein, baicalin, and wogonin (21, structures shown in Fig. 3A). We therefore screened these 3 flavones for IRF-1 activity using both IRF-1–luc/AGS and IRF-1–luc/MDA468. In both systems, we found that baicalein showed the most potent induction of IRF-1 activity. Baicalein at a concentration of 0.5 μmol/L resulted in a significant increase in IRF-1–dependent luciferase activity compared with control (P = 0.0063 and P = 0.0004 in AGS and MDA468, respectively) and increased normalized luciferase activity to approximately 3-fold over no treatment at 5 to 10 μmol/L, markedly higher than any concentration of the other flavones (Fig. 3A). Furthermore, when evaluating for IRF-1 protein by immunoblot, increased protein level is identified at 10 μmol/L and is markedly increased at 25 and 50 μmol/L for baicalein, with no consistent increase with baicalin and perhaps even decrease of IRF-1 protein with wogonin in the AGS cell line (Fig. 3B). We then conducted dose- and time-course studies of the induction of IRF-1 protein by immunoblotting in baicalein-treated AGS cells and found that once again there appears to be an increase in protein levels at 10 μmol/L at 24 hours in a dose-related fashion and an increase in IRF-1 at 12 hours that increases significantly by 48 and 72 hours. Previously, we showed that IRF-1 upregulates PUMA, which mediates mitochondrial apoptosis in cancer cells (17), and in Figure 3C, we show that PUMA does increase in concert with IRF-1 in baicalein-treated AGS cells. Previously, IRF-1 has been shown to mediate its tumor-suppressive effect by downregulation of cyclin D1 in transformed cells (8), and we found that cyclin D1 decreases in concert with IRF-1 in baicalein-treated cells (Fig. 3C). Finally, as PUMA is also known to be induced by p53, we treated the p53-mutant human breast cancer cell line BT549 with baicalein and confirmed an increase in IRF-1 and an increase in PUMA independent of p53 (Fig. 3D), similar to results we had previously published with Ad-IRF-1 and tetracycline-inducible IRF-1 cells in p53-mutant or -deleted cells (17). We also examined cell lysates for mRNA levels of IRF-1 by quantitative real-time RT-PCR and found significantly increased IRF-1 mRNA levels in AGS cells treated with baicalein in the approximately 50% to 100% range (P = 0.01–0.0008), implicating a transcriptional...
component to the enhanced IRF-1 protein and activity (Supplementary Data).

**Baicalein inhibits growth of cancer cells in vitro**

The rationale for screening for enhancers of IRF-1 activity was based on evidence that IRF-1 causes tumor suppression of cancer cells. This is further supported by data here showing upregulation of PUMA and downregulation of cyclin D1 (Fig. 3D). We therefore examined the effect of SB extract and its active components on growth of AGS cells *in vitro*. As shown in Figure 4A, SB exerted a dose- and time-dependent inhibition on AGS cell growth. Among the active components of SB, the MTT assay showed that baicalein inhibited the growth of AGS cells to the greatest extent (Fig. 4B). Baicalin had no inhibitory effect on cell growth (Fig. 4C), and wogonin had significantly less growth inhibition (Fig. 4D).

**Baicalein inhibits growth of cancer cells in vitro, which is in part mediated by IRF-1**

We also investigated the antiproliferative effect of baicalein on the human breast cancer cell lines SKBR3 and MDA468. MTT assays show that baicalein can inhibit cell growth significantly in both cell lines in a dose-dependent manner, with IC₅₀ values between 1 and 10 μmol/L, whereas baicalin has minimal effect (Fig. 5A and B). We have already shown enhanced IRF-1 activity induced by baicalein in AGS and MDA468 cells (Fig. 3A) and have also confirmed this by luciferase assay in SKBR3 (Supplementary Data). We wished to use RNA interference technology to see whether knockdown of IRF-1 expression would decrease the growth inhibitory response by baicalein. We were unable to sufficiently inhibit the induction of IRF-1 protein by baicalein in AGS cells treated with short interfering RNA transfection or clones that express short hairpin

![Graphs and images related to the text content...](image-url)
Baicalein inhibits cancer cell growth in vivo

Baicalein can inhibit cell growth in different cancer cell lines in vitro; however, there has been little data on its effect on tumor growth in vivo. We conducted studies on a xenogeneic model using AGS cells. Tumor-bearing SCID-Bg mice were treated with a carrier, 20 mg/kg baicalein, or 20 mg/kg genistein through i.p injection 5 times per week. Genistein was chosen as a comparison control because it is a flavonoid-like baicalein and has the same chemical formula but has a different structure. As shown in Figure 6A, baicalein inhibits tumor growth in this model in comparison with either carrier or genistein whereas genistein has no significant difference with carrier. There was no evidence of toxicity in any of the treated mice despite repeated doses. To prove that baicalein can inhibit tumor growth in a syngeneic mouse model, we used C3H/HeJ mice transplanted with the mouse breast cancer cell line C3L5, which we have shown previously to have marked tumor suppression by IRF-1 expression in vivo (14). We first confirmed that baicalein can enhance IRF-1 activity and inhibit cell growth in this cell line (Fig. 6B and C). Systemic administration of baicalein to C3L5 tumor-bearing C3H/HeJ mice resulted in significant growth inhibition of these mouse breast tumors (Fig. 6D), with no evidence of toxicity in these animals. Taken together, these results provide strong evidence for the tumor growth-inhibiting activity and tolerability of baicalein in vivo.
Discussion

A cell-based screening strategy was previously reported for identifying enhancers of STAT-1 activity, which identified a compound that enhanced IFN-γ-induced STAT-1-dependent gene expression (18). This strategy had the advantage of rapidly excluding compounds that were not cell permeable and displaying nonspecific toxicity. Furthermore, it allowed for the identification of active compounds independent of a predetermined mechanism of action, which, in turn, allowed for the identification of compounds that may function by a variety of mechanisms and may reveal levels of functional regulation that might not have been previously appreciated (18). Our screening strategy used a similar approach, making use of the same advantages. However, cancer cells may downregulate STAT-1 as seen in AGS and other cancer cells (22). Furthermore, STAT-1 activity has been found to have protumorigenic activities in certain contexts (19) and can also confer resistance to radiation and conventional cytotoxic agents (23). Focusing on a target farther downstream may circumvent signaling pathways that are protumorigenic or confer treatment resistance. Notably, IRF-1 does not seem to be upregulated in radio-resistant and other resistant cancer cells, despite significant expression of STAT-1 (19).

Significant IRF-1 enhancing activity of baicalein is seen at concentrations in the low micromolar range, with dose-dependent increase in IRF-1 protein and cell growth inhibition. We purposely limited our study to concentrations of 50 μmol/L or lower, as these concentrations are achievable in vivo, based on multiple studies of the pharmacokinetics of flavonoids including baicalein. Studies of flavonoids in humans have shown that dietary consumption of foods rich in flavonoids can result in plasma levels of individual flavonoids in the single-digit micromolar range. For example, women who consume varying amounts of flavonoids from soy milk have plasma genistein levels of 0.8 to 2.2 μmol/L (24). After repeated injections of baicalein intraperitoneally into mice at 20 mg/kg for 5 days per week, there was no evidence of toxicity. Furthermore, baicalein has been ingested by humans in human studies and in TAM, supporting the hypothesis that baicalein may be well tolerated in humans with plasma levels comparable with those used in our study (21).

The determination of the mechanism of enhancement of IRF-1 activity by baicalein requires further study. We evaluated mRNA transcript for IRF-1 by quantitative real-time RT-PCR and found consistent increases in transcript in the AGS cell line when compared with retinoic acid as a positive control in the approximately 50% to 100% range (Supplementary Data). However, the increases in protein seem to reach approximately 550% at the same doses, although it is difficult to make such comparisons. We hypothesize that baicalein also affects the rate of protein turnover of IRF-1 in certain cancer cells that results in persistence of IRF-1 protein and increased transcriptional activity. The IRF-1 protein normally has a half-life of approximately 30 minutes. Treatments such as ionizing radiation have already been found to increase steady-state levels of the IRF-1 protein through a concerted mechanism that includes increases in IRF-1 transcription and decreases in the rate of degradation (25). IRF-1 is known to be degraded by the ubiquitin/proteasome pathway (26). Baicalein may interfere with this process, perhaps through the same mechanism as ionizing radiation or DNA-damaging agents. Indeed, numerous studies have shown the potential role of reactive oxygen species in baicalein-induced cellular effects, including apoptosis (27).

It is notable that shRNA against IRF-1 attenuated, but did not abrogate, the tumor suppression caused by baicalein as assessed by MTT assay (Fig. 5C). This may be due to incomplete suppression of IRF-1 protein by the shRNA, as an IRF-1 band was still visible in our best shRNA clone, and small amounts of detected protein may have significant activity. Standard transient transfection techniques with short interfering RNA to keep IRF-1 protein low in the presence of baicalein proved to be very difficult, as baicalein may affect IRF-1 protein expression at multiple levels. Regardless, there may be multiple off-target effects of baicalein not related to IRF-1 activity. Baicalein has been found to inhibit 12-lipoxygenase activity (28–30) as well as increase the TRAIL receptor DR5 (27). Although baicalein was identified by screening for IRF-1 activity, IRF-1 certainly may not be the sole mediator of in vitro growth inhibition. This does not diminish the potential for this assay to identify useful compounds in cancer therapy.

In conclusion, we used a cell-based screening system of natural extracts and their active components to identify a compound that can enhance IRF-1 activity and cause tumor suppression of cancer cells in vitro and in vivo. Through further mechanistic and preclinical studies, we will be able to determine the potential clinical uses of this natural compound or its parent extract in cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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