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Preclinical in vitro, in vivo and pharmacokinetic evaluations of FLLL12 for the prevention and treatment of head and neck cancers

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Abstract

Despite its high promise for cancer prevention and therapy, the potential utility of curcumin in cancer is compromised by its low bioavailability and weak potency. The purpose of the current study was to assess the in vitro and in vivo efficacy and pharmacokinetic parameters of the potent curcumin analog FLLL12 in SCCHN and identify the mechanisms of its anti-tumor effect. IC₅₀ values against a panel of one premalignant and eight malignant head and neck cancer cell lines as well as apoptosis assay results suggested that FLLL12 is 10–24-fold more potent than natural curcumin depending on the cell line and induces mitochondria-mediated apoptosis. In vivo efficacy (xenograft) and pharmacokinetic studies also suggested that FLLL12 is significantly more potent and has more favorable pharmacokinetic properties than curcumin. FLLL12 strongly inhibited the expression of p-EGFR, EGFR, p-AKT, AKT, Bcl-2 and Bid and increased the expression of Bim. Overexpression of constitutively active AKT or Bcl-2 or ablation of Bim or Bid significantly inhibited FLLL12-induced apoptosis. Further mechanistic studies revealed that FLLL12 regulated EGFR and AKT at transcriptional levels, whereas Bcl-2 was regulated at the translational level. Finally, FLLL12 strongly inhibited the AKT downstream targets mTOR and FOXO1a and 3a. Taken together, our results strongly suggest that FLLL12 is a potent curcumin analog with more favorable pharmacokinetic properties that induces apoptosis of head and neck cancers.

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cancer cell lines by inhibition of survival proteins including EGFR, AKT and Bcl-2 and increasing of the pro-apoptotic protein Bim.

**Keywords**

Head and neck cancer; apoptosis; curcumin; protein translation; signal transduction

**Introduction**

Although cancer is a devastating disease and limited options are available for the treatment of advanced stage disease, curative treatment options are available for most cancers if detected early. However, the emergence of resistance to therapy, drug-associated toxicities, recurrence and the development of second primary tumors are the major hurdles that continuously challenge therapeutic success (1, 2). Unlike chemotherapy drugs, the safety of many natural compounds, including curcumin, has been well established through centuries of human consumption (3, 4).

Although curcumin exhibits potential anti-tumor and chemopreventive effects in vitro and interferes with multiple oncogenic and tumor suppressor pathways, its clinical application is severely compromised by its poor absorption, low bioavailability, rapid biotransformation and low potency (5, 6). To circumvent these issues, approaches such as the synthesis of more potent and bioavailable analogs and the modification of delivery systems have been extensively considered. The β-diketone moiety is responsible for the instability and weak pharmacokinetic profile of curcumin. Structural modifications of the aryl side chains or diketone moiety have significantly improved solubility, stability, and bioavailability (7). More than a thousand monocarbonyl analogs of curcumin have been synthesized and tested in vitro and in vivo for their anti-cancer effects. Many of these compounds show 10–20–fold more potency than curcumin, have better pharmacokinetic properties and effectively inhibit xenograft growth (8). GO-Y078, 079, 030, 097, and 098 comprise a group of analogs that are more soluble in water and are at least 10-fold more potent than natural curcumin (9). Several members of the EF-series of curcumin analogs, including EF24, 31 and UBS109, synthesized by modifying the diketo chain showed ~10-fold better anti-cancer efficacy than natural curcumin in vitro and inhibited tumor growth in xenograft models (10–12). Many members of the FLLL-series of analogs synthesized by modifying the aryl side chain also exhibited higher anti-growth efficacy and selectivity for cancer cells sparing normal cells (13, 14). FLLL32 also significantly inhibited breast cancer xenograft growth in nude mice (15). Dimethoxycurcumin exhibited significantly higher stability in vivo and against microsomal metabolism (16). PAC, another synthetic curcumin analog, showed higher stability in blood and greater biodistribution and bioavailability than curcumin in mice and is more water soluble (17). The compound is also more potent in vivo in inducing apoptosis. FH Sarkar’s group has synthesized a series of curcumin analogs and evaluated their effects against colon and pancreatic cancer cell lines (18). The group has identified a compound known as CDF with superior anti-cancer activity in colon, prostate and pancreatic cancer cell lines that exhibited 2.7-fold greater systemic drug level and 10.6-fold higher accumulation in
pancreatic tissue than curcumin (19). Vyas et al. and Park et al. have comprehensively reviewed curcumin analogs with improved efficacy and bioavailability (5, 7).

In the current study, we investigated the pharmacokinetic properties, the in vivo and in vitro anti-tumor efficacy, and the mechanism of apoptosis induced by FLLL12 in squamous cell carcinoma of the head and neck (SCCHN). SCCHN is the 6th most common cancer in the US and represents ~3% of all cancer cases, with an estimated 59,000 new cases and 12,000 deaths in 2014 (20). FLLL12 is a synthetic curcumin analog synthesized by modifying the aryl side chains to circumvent the efficacy, selectivity and bioavailability issues associated with natural compounds. FLLL12 is ~10-fold more potent than natural curcumin against breast, prostate, colorectal, pancreatic and lung cancer cell lines and possesses selective activity against cancer cells (13, 21–23). FLLL12 induces apoptosis of these cancer cells by inhibition of two major survival pathways, AKT and STAT3 or inducing DR5 expression. However, the detailed mechanisms underlying FLLL12-induced apoptosis are not fully understood. Moreover, FLLL12 has never been tested in vivo or against SCCHN cancer cell lines. The pharmacokinetic (PK) properties of FLLL12 are also unknown. In the present study, for the first time, we showed that depending on the cell line, FLLL12 is 10–24-fold more potent than curcumin and induces apoptosis in SCCHN cell lines in vitro. Moreover, we demonstrated that FLLL12 induces apoptosis in SCCHN by modulation of multiple Bcl-2 proteins and transcriptional downregulation of EGFR and AKT. We also showed that FLLL12 has 3–4-fold more favorable C\text{max} and AUC in mice than curcumin and is significantly more potent in inhibiting tumor volume in a SCCHN xenograft model. Since premalignant cells are more sensitive than malignant cells, the compound might be well suited for chemoprevention.

Material and Methods

Cell lines

- **Cell lines**—The Tu212 and Tu177 cell lines were established from hypopharyngeal tumor and poorly differentiated squamous carcinoma of the larynx, respectively and were kindly provided by Dr. Gary L. Clayman (University of Texas MD Anderson Cancer Center, Houston, TX) in 2002. The MDA886LN cell line was derived from lymph node metastasis of squamous cell carcinoma of the larynx and was procured from Dr. Peter G Sacks’ laboratory in 2002 when he was at the MD Anderson Cancer Center. MDA686TU (Tu686) was established from primary tongue cancer. The head and neck premalignant cell line MSK-Leuk1 (MSK) was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue and maintained in keratinocyte basal media (24). These cell lines were gifts from Dr. Peter G. Sacks (New York University College of Dentistry, New York, NY) and procured in 2014 and 2012, respectively. The JHU022 cell line was established from the lymph node metastasis of squamous cell carcinoma of the larynx. PCI-13 and SqCCY1 were established from primary oral cavity cancers and UM-22B from the lymph node metastasis of a hypopharyngeal cancer. PCI-13, UM-22B and JHU022 cell lines were procured from Dr. Robert Ferries’ laboratory (University of Pittsburgh) in 2012. SqCCY1 was obtained from Dr. Shi-Yong Sun at Emory University (Atlanta, GA) in 2012. The SCCHN cell lines were maintained in DMEM/F12 (1:1) medium.
supplemented with 10% heat-inactivated fetal bovine serum in a 37 °C, 5% CO₂ humidified incubator. The authenticity of all these cell lines was verified through the genomic short tandem repeat (STR) profile by the Research Animal Diagnostic Laboratory, University of Missouri (Columbia, MO) in September 2009, and by the Emory University Integrated Genomics Core (EIGC) in October 2013, respectively.

**Reagents**

Curcumin was purchased from Sigma Chemicals (St Louis, MO, USA) and FLLL12 was obtained from Dr. James R. Fuchs’ laboratory (Ohio State University, Columbus, USA). FLLL12 and curcumin were dissolved in DMSO and preserved as stock solutions for *in vitro* studies. During experiments, the reagents were further diluted directly in a cell culture dish with RPMI medium. The final concentration of DMSO was <0.1%.

**Measurement of IC₅₀**

Appropriate numbers of cells were seeded with 100 µL medium in 96-well culture plates and incubated overnight before treatment with FLLL12 or curcumin. The cells were treated with various concentrations of FLLL12 or curcumin and incubated for an additional 72 h. Inhibition of cell growth was determined by SRB assay as described elsewhere (25). IC₅₀ value was calculated by using CalcuSyn software (Biosoft, UK).

**Western blot analysis**

Whole cell lysates were extracted from cells using lysis buffer. The protein concentration of each sample was determined by protein assay kit (Bio-Rad, CA, USA). Equal amounts of protein (20 µg) from each sample were separated on 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA) and incubated with appropriately diluted specific primary antibodies. Mouse anti–β-actin (Sigma) or rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Trevigen, MD, USA) antibody was used as a sample loading control. Immunostained protein bands were detected with an enhanced chemiluminescence kit (Pierce, IL, USA).

**Annexin V-phycoerythrin staining for apoptosis**

Cells were seeded at a concentration such that they were 40–50% confluent at the time of treatment and treated with different concentrations of FLLL12 and curcumin for the indicated time, then trypsinized and washed in cold 1× PBS. The cells were then resuspended in 1× Annexin V binding buffer (BD PharMingen), and stained with Annexin V-phycoerythrin (Annexin V-PE; BD PharMingen) and 7-AAD (BD PharMingen) for 15 min at room temperature. The stained samples were measured using a fluorescence-activated cell sorting caliber bench-top flow cytometer (Becton Dickinson). FlowJo software (Tree Star) was used for apoptosis analysis.

**Real time qPCR analysis**

Total RNA was extracted from cells using an RNeasy mini kit (Qiagen, Valencia, CA, USA). A total of 2 µg of RNA was reverse transcribed to cDNA using a cDNA synthesis kit.
(Bio-Rad, CA, USA) according to the manufacturer’s protocol. Quantitative real time PCR (qPCR) was carried out by First SYBER Green Master Mix (Applied Biosystem, NY, USA) according to the manufacturer’s instructions. Primers used for qPCR are listed in the supplementary materials.

**siRNA transfection**

Bid-specific siRNA were purchased from Qiagen (Valencia, CA, USA) and non-targeting control siRNA was obtained from Dharmacon (Chicago, IL, USA). Cells were seeded in 6-cm plates 24 h before transfection in medium containing 5% FBS, so that they reached 30% to 50% confluency. siRNA was complexed with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions and applied to each plate. Transfection media was removed and replaced with fresh media after 6 h of transfection. Knockdown efficiency of each target gene was evaluated by Western blotting after 48 h of transfection. Retroviral transduction of shBim was described elsewhere (26).

**Pharmacokinetic studies**

Female A/J mice (Harlan) weighing 20 to 25 grams were used for the pharmacokinetic studies. The experiment was approved by the Animal Care and Use Committee of Emory University (DAR-2002195-021516BN). Mice were maintained on Bed-o’Cobs bedding in temperature (22 ± 2 °C) and humidity (30–50%) controlled rooms with a 12 hour light/dark cycle. Rodent Chow No. 5001 (LabDiet, St. Louis, MO) and autoclaved water were provided ad libitum. Mice were given a single dose of FLLL12 or curcumin by oral gavage (200 mg/kg) in 10% DMSO/0.5% CMC. 2–3 mice were sacrificed at each time point (0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 24 h) and ~0.3 ml of blood was taken immediately from each animal by cardiac puncture. Samples were collected in heparinized capillary tubes and centrifuged within a few minutes at 2000 × g for five minutes in a refrigerated centrifuge to obtain plasma. Plasma was transferred to amber Eppendorf tubes on ice, frozen for one hour and stored at −80°C until analysis by LC/MS/MS. The LC/MS/MS analysis was conducted at Agilux Laboratories (Boston, MA). Protein precipitation was used to remove plasma proteins. Aliquots of 10 µL of samples, matrix calibration standards and blank controls were added into a 96-well plate. 60 µL of internal standard was added to all samples except matrix blanks. 60 µL of acetonitrile was added to the matrix blanks. After centrifugation (3,000 rpm for 3 min) at ambient temperature, 50 µL of supernatant was transferred to a clean 96-well plate and 100 µL of water was added. Separation of curcumin and FLLL12 was achieved using UPLC BEH C18 1.7 µM column at 40°C by gradient elution utilizing the following profile: 0–0.25 minute 70% A and 30% B, 0.25–2 minutes 10% A and 90% B. Mobile phase A consists of H2O:acetone:formic acid 95:5:0.1 (v/v/v) and mobile phase B consists of MeOH:acetone:formic acid 50:50:0.1 (v/v/v). The flow rate was 0.9 ml/min. API-6500 was used for MS/MS. FLLL12 detection was accomplished using multiple reaction monitoring (MRM) in positive electrospray ionization mode with parent ion of 387.1 m/z, daughter ion 175.1 m/z. Curcumin detection was accomplished with parent ion of 369.1 m/z, daughter ion 177.1 m/z. Carbamazepine (parent ion 237.1 m/z, daughter ion 194.1 m/z) and glyburide (parent ion 494.2 m/z and daughter ion 369.1 m/z) were used as internal standards for FLLL12 and curcumin, respectively. Spectra and chromatograms of the compounds were acquired and processed using the Analyst® Version 1.6.2 (Applied Biosystems Sciex).
Standard curves were constructed using peak area ratio (Y) against the corresponding nominal concentrations (X, ng/mL). The LC-MS/MS assay was also validated with specificity, precision (<15%), accuracy (>85%) and linearity (1 to 5000 ng/mL, r>0.99). PK parameters were calculated using Kinetica™ software (5.0, Thermo Fisher, Scientific, Chelmsford, MA). Half-life values were estimated by compartmental analysis, while area under the plasma concentration-time curve (AUC) and clearance were estimated by non-compartmental analysis of the mean concentration values.

Nude mouse xenograft model

The animal experiments were approved by the Animal Care and Use Committee of Emory University (DAR-2002630-050517BN). Eighteen female nude mice (athymic nu/nu, Taconic, NY), aged 4–6 weeks (~20 g weight), were used for the study. Mice were maintained on Alpha-Dri bedding in temperature (22 ± 2 °C) and humidity (30–50%) controlled rooms with a 12 hour light/dark cycle. Rodent Chow No. 5010 (LabDiet, St. Louis, MO) and autoclaved water were provided ad libitum. After adaptation for a few days in the new environment, the mice were subcutaneously injected with 4×10^6 Tu686 cells into the right flank. After about a week when visible tumors had formed, the mice were randomly divided into three groups. Each mouse was intraperitoneally treated with vehicle control (n=6), curcumin (50 mg/kg, n=6), or FLLL12 (50 mg/kg, n=6) 5 days a week. The tumor size (larger diameter and smaller diameter) was measured 2 times a week using a digital caliper. The tumor volume was calculated using the formula: 

\[ V = \pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2, \]

as reported previously (27). Growth curves were plotted using average tumor volume within each experimental group at the set time points.

Statistical analysis—Experimental values were represented as mean ± standard deviation in triplicate. The significance of differences was determined by the Student’s t-test. A value of \( p<0.05 \) was considered statistically significant. For analysis of tumor growth, the mixed effects model was implemented to estimate and compare the tumor volume among the experimental groups, in which the correlation among the repeated measurements in each mouse over time was accounted for accordingly. The log transformed was also applied to tumor volume to meet the normality and equal variance assumption, and p-value was adjusted for multiple comparisons.

Results

FLLL12 is more potent than curcumin and induces intrinsic apoptosis

Although the antitumor effects of FLLL12 have been investigated in prostate, breast, pancreatic, lung and colon cancer cell lines and compared with those of curcumin (13, 21–23), the agent has never been tested in SCCHN cell lines. In order to explore the mechanism of anti-tumor effect of FLLL12 in SCCHN, we first assessed the sensitivity of different premalignant (MSK-LEUK1) and malignant SCCHN cell lines to FLLL12 versus curcumin by comparing IC_{50} values measured using SRB assays at 72 h. As shown in Table 1, depending on the cell line, the IC_{50} values of FLLL12 ranged from 0.35–1.55 µM, compared with 4.53–17.42 µM for curcumin. These results suggest that FLLL12 is 10–24-fold more potent than curcumin, depending on the cell line. Since induction of apoptosis is critical for
effective tumor regression and elimination of premalignant cells, we next measured apoptosis by annexin V-PE staining. As shown in Figure 1 (A-E), FLLL12 dose- and time-dependently induced apoptosis in SCCHN cell lines. While 1–3 µM of FLLL12 was sufficient to induce ~80% apoptosis by 48 h, ~10–15 µM of curcumin was required to induce comparable apoptosis. The doses for apoptosis assays were selected based on IC50 values, thus varied with cell lines. For UM-22B and MSK cell lines, equimolar doses of FLLL12 and curcumin were used to show that curcumin was ineffective at lower doses. A single time point experiment was conducted for the MSK cell line since 24 h treatment yielded more than 80% apoptosis. Further increase in treatment time might not produce accurate results. These results further confirm that FLLL12 is 5–10-fold more potent than curcumin against SCCHN cell lines in inducing apoptosis. Finally, to confirm apoptosis induction by FLLL12, we assessed the cleavage of PARP, which is considered a marker of apoptosis in response to apoptotic signaling, in one premalignant and two malignant cell lines. Treatment of cells with FLLL12 markedly induced the cleavage of PARP in these cell lines (Figure 2A–C). Apoptosis mechanisms involve either mitochondria-mediated intrinsic or death receptor-mediated extrinsic pathways. To identify the mechanism of FLLL12-induced apoptosis, we analyzed cytochrome c release in the cytoplasm, which is indicative of mitochondria-mediated apoptosis, after treatment with FLLL12. As shown in Fig. 2D, FLLL12 caused release of cytochrome c in the cytoplasm. ERK and Cox4 were used for cytoplasmic and mitochondrial controls, respectively. The levels of Cox4 in FLLL12 and curcumin treated cells were decreased, probably due to massive apoptosis induction in these cells. This result suggests that FLLL12 induces mitochondria-mediated apoptosis.

Role of Bcl-2 proteins in FLLL12-induced apoptosis

The Bcl-2 family proteins are the mediator of mitochondria-mediated intrinsic apoptosis, and are essential for maintaining MOMP. Since FLLL12 induced mitochondria-mediated apoptosis, we next examined the expression of the anti-apoptotic Bcl-2 proteins Bcl-2, Bcl-xL and Mcl-1, and pro-apoptotic Bcl-2 proteins Bim and Bid after treatment with FLLL12. We found that the expression of pro-apoptotic Bim and Bid and anti-apoptotic Bcl-2 were modulated by FLLL12. While the expression of Bcl-2 and Bid (full length) were inhibited, Bim expression was increased by FLLL12 (Fig. 3A). The expression of Bcl-xL and Mcl-1 remained mostly unchanged. Among these changes, the modulation of Bcl-2, Bim and Bid favored the induction of apoptosis. To study the role of inhibition of Bcl-2 in FLLL12-induced apoptosis, we overexpressed Bcl-2 in Tu686 cells via retroviral transduction, established a pool of cells overexpressing Bcl-2 by G418 selection (28) and analyzed apoptosis in these cells after FLLL12 treatment. As shown in Fig. 3B, overexpression of Bcl-2 significantly protected cells from FLLL12-induced apoptosis. To study the mechanism of inhibition of Bcl-2 by FLLL12, we assessed the expression of Bcl-2 mRNA (Fig. 3C). Interestingly, the expression of Bcl-2 mRNA was increased rather than decreased after FLLL12 treatment in two different cell lines, suggesting that the regulation is either at the translation or post-translational level. We also ruled out the possibility of increased post-translational degradation of Bcl-2 by FLLL12, since pretreatment with the proteasome inhibitor MG132 failed to rescue Bcl-2 expression (Fig. S1A). Finally, we assessed the expression of Bcl-2 protein after inhibition of global protein synthesis with cycloheximide. FLLL12 had no further effect on Bcl-2 protein expression (Fig. S1B). Results presented in
Fig. 3C and S1 thus suggest that FLLL12 possibly inhibited the expression of Bcl-2 at the translational level, since FLLL12 failed to further increase or decrease Bcl-2 expression after protein translation was shut down, an indication that cyclohexamide and FLLL12 have parallel effects on Bcl-2 expression. We also studied the role of Bim by knocking down its expression using siRNA. As shown in Fig. 3D, ablation of Bim expression significantly protected cells from FLLL12-induced apoptosis. The efficiency of Bim knockdown and cleavage of PARP are shown in Fig. S2A, B. We also examined the role of Bid in apoptosis by knocking down its expression. Bid is a pro-apoptotic Bcl-2 protein activated by active caspase 8 through truncation which inhibits expression of the full length form. As shown in Fig. 3E, ablation of Bid also significantly protected cells from FLLL12-induced apoptosis. Activation of caspase-8 also supported that inhibition of Bid expression was due to truncation (Fig. S3A). The efficiency of Bid knockdown and PARP cleavage are shown in Fig. S3B.

Inhibition of EGFR and AKT transcription by FLLL12

Activation of EGFR is one of the early molecular events in SCCHN carcinogenesis (29). Since both curcumin and FLLL12 were previously reported to inhibit EGFR and AKT activation, we next examined the activation of EGFR and AKT by measuring the level of phosphorylated proteins in one premalignant and two malignant cell lines. As shown in Fig. 4A, while FLLL12 and curcumin both inhibited the phosphorylation of EGFR and AKT, FLLL12 did so with much greater potency than curcumin. Interestingly, FLLL12 and curcumin not only inhibited phosphorylated EGFR and AKT, but also total EGFR and AKT proteins. We next examined the importance of AKT inhibition by overexpressing constitutively active (CA)-AKT. A pool of cells overexpressing CA-AKT via retroviral transduction was generated as described (28). Functional characterization of these cells is shown in Fig S4. Activation of p-S6 after transduction of CA-AKT suggests that the exogenous AKT is functionally active. These cells were used to measure apoptosis after FLLL12 treatment. As shown in Fig. 4B and C, overexpression of CA-AKT significantly protected cells from FLLL12-induced apoptosis (p<0.05) and inhibited PARP cleavage and caspase-3 activation. We also examined the expression of Bim and Bcl-2 in these cells. The FLLL12-induced increase in Bim expression was strongly inhibited in cells overexpressing CA-AKT (Fig. 4C). On the other hand, the CA-AKT overexpressing cell line had a higher basal level of Bcl-2 and FLLL12-induced inhibition of Bcl-2 expression was less pronounced in these cells as compared to vector transduced control cells (Fig. 4C). Bim is a known transcriptional target of FOXO transcription factors, which are negatively regulated by AKT. Thus, it is not surprising that inactivation of AKT activated FOXO-dependent transcription.

In order to understand the kinetics of the inhibition of AKT and phosphorylated AKT, we assessed the expression of phosphorylated AKT and AKT at 12h. As shown in Fig. S5A, although the expression of phosphorylated AKT was inhibited at 12h treatment, there was no remarkable change in the expression of total AKT suggesting that inhibition of phosphorylated AKT preceded inhibition of total AKT. To test whether the inhibition of EGFR and AKT phosphorylation plays any role in the inhibition of corresponding protein, we examined the expression of EGFR and AKT proteins after pretreating cells with the
EGFR inhibitor erlotinib and PI3K inhibitor LY294002. No significant changes in the expression of EGFR and AKT proteins were observed after pretreatment with the corresponding inhibitors. FLLL12 was able to inhibit AKT and EGFR even after pretreatment with the inhibitors (Fig. S5B, lanes 3 and 5). On the other hand, EGFR and AKT inhibitors had no effect on the expression of these proteins after a total 25 h treatment (Fig. S5B, lanes 4 and 6). These results suggest that although inhibition of phosphorylation is an early event, it is not important for the inhibition of protein expression. We also found that pretreatment with the proteasome inhibitor MG132 failed to rescue the expression of EGFR and AKT proteins (Fig. S5C). These results ruled out the possibility of proteasome-mediated posttranslational degradation of EGFR and AKT. We also assessed the expression of EGFR and AKT proteins after inhibition of global protein synthesis. However, FLLL12 had no remarkable effect on the expression of EGFR and AKT proteins once global protein synthesis was shut down (Fig. S5D). These results further excluded the possibility of posttranslational regulation of EGFR and AKT by FLLL12. If FLLL12 regulates EFGR and AKT at the posttranslational level, we would see modulation of EGFR and AKT protein expression after the inhibition of protein synthesis or the proteasome. To confirm transcriptional or translational regulation of EGFR and AKT by FLLL12, we assessed the expression of EGFR and AKT mRNA by real-time qPCR. As shown in Fig. 4D and 4E, FLLL12 as well as curcumin strongly inhibited the mRNA expression of both EGFR and AKT. Taken together, the data presented in Fig. 4 and S5 demonstrate that FLLL12 regulates EFGR and AKT at the transcriptional level.

Inhibition of AKT downstream targets by FLLL12

Activation of the EGFR-AKT pathways impacts a number of downstream cell survival pathways. One such pathway is the mTOR-mediated protein translational pathway. We next examined the phosphorylation of mTOR, S6 and 4EBP1 as readouts for the mTOR pathway. As shown in Fig. 4F, treatment with FLLL12 strongly inhibited phosphorylation of both axes of the mTOR pathway, i.e. phosphorylation of both S6 and 4EBP1. The FOXO family of transcription factors is another important downstream target of AKT that plays a critical role in cell survival and apoptosis and regulates the expression of Bim. We also assessed the phosphorylation of FOXO proteins after FLLL12 treatment and found that FLLL12 also strongly inhibited the phosphorylation of FOXO1 and 3 at multiple sites (Fig. 4G).

In vivo efficacy and pharmacokinetic properties of FLLL12

The oral bioavailability of curcumin is ~1% (30, 31), making it a significant challenge to attain an effective concentration of 15–30 µM in vivo. To investigate whether FLLL12 has better pharmacokinetic parameters than curcumin, we measured plasma concentrations of both curcumin and FLLL12 in A/J mice using LC/MS/MS (Fig. 5A and B). Standard curves are shown in Fig. S6A and B. After oral administration of a dose of 200 mg/kg in a DMSO/0.5% CMC (10%/90%) formulation, peak mouse plasma concentrations were reached at 0.25 and 0.5 hours post-dose (T_{max}) with average concentrations (C_{max}) of 55.65 and 241.5 ng/mL for curcumin and FLLL12, respectively. These results suggest that the C_{max} of FLLL12 is 4.3-fold greater than that of curcumin. The terminal elimination half-lives (t_{1/2}) were 4.8 and 7.7 hours, with an average AUC (0-∞) of 418.5 and 131 hr x ng/mL for FLLL12 and curcumin, respectively. The mean concentration versus time profiles suggested
that the compounds were quickly absorbed following oral administration, clearance from blood occurred at a moderate rate following \( C_{\text{max}} \) and FLLL12 had more favorable pharmacokinetic profile than curcumin. Since curcumin undergoes extensive metabolism, we paid special attention to the chromatograms for extra peaks. Like curcumin, we also detected some extra peaks in the chromatograms of FLLL12 which were absent in the blanks and standard samples as early as 0.25 h, suggesting that FLLL12 might also undergo rapid metabolism (Fig. S7). However, the number of extra peaks for FLLL12 was less than that of curcumin, which suggests that fewer metabolites of FLLL12 are formed. However, more detailed studies are required to identify these metabolites and confirm the finding.

To investigate the \textit{in vivo} efficacy of FLLL12 and curcumin, animals bearing Tu686 SCCHN xenografts were treated with vehicle (0.5% CMC and 10% DMSO in water; \( n=6 \)); 50 mg/kg FLLL12 (\( n=6 \)) and 50 mg/kg curcumin (\( n=6 \)) in a formulation containing the vehicle. Drugs were administered via intra-peritoneal injection, once/day, five days/week (M-F) for 17 days. As shown in Fig. 5C, treatment with FLLL12 led to significantly smaller tumors than with vehicle or curcumin treatment (\( p<0.05 \), Supplementary Table S1). Although FLLL12 significantly reduced tumor volume, the same dose of curcumin had no effect, suggesting that FLLL12 is also more potent than curcumin \textit{in vivo}. We also measured body weight throughout the study. No significant changes in body weights were observed among the various groups (Fig. 5D). H&E staining of the major organs collected at the end of the study also suggested no major organ-related toxicities (Fig. S8). A board-certified pathologist carefully examined the organs of all mice from the xenograft studies for signs of toxicity. Taken together, our findings from these animal studies suggest that FLLL12 is more effective in inhibiting tumor growth than curcumin without inducing any notable toxicity in general.

**Discussion**

Curcumin has been extensively investigated for the last few decades in clinical and preclinical settings for its potential anti-tumor and chemopreventive effects. Although preclinical investigations were encouraging, clinical studies were disappointing due to the poor bioavailability of curcumin (31). On the other hand, the structure of curcumin makes it an excellent lead compound for structural modifications. Dozens of analogs have been synthesized so far with better efficacy and bioavailability (5). In the present study, we investigated the \textit{in vivo} efficacy, pharmacokinetic properties and \textit{in vitro} mechanism of anti-tumor effects, particularly of apoptosis, of a curcumin analog FLLL12 in SCCHN cell lines. To our knowledge, this is the first \textit{in vivo} efficacy and pharmacokinetic study using FLLL12. Also, this is the first detailed mechanistic study and first testing in SCCHN cells of this analog. Our findings clearly demonstrate that FLLL12 has much more favorable pharmacokinetic properties than the parent compound, with ~4.0 and ~3.5-fold greater \( C_{\text{max}} \) and AUC, respectively. Consistent with other studies, the concentration versus time graph suggests that both FLLL12 and curcumin follow a reabsorption/secondary absorption phase in mice after an initial decline in serum concentration (19, 32). FLLL12 is also effective in reducing tumor volume \textit{in vivo} at a dose of 50 mg/kg and exhibited significantly better efficacy than curcumin. Previously, we conducted an \textit{in vivo} xenograft study using 150 mg/kg of curcumin, and did not find any efficacy of curcumin at this dose level (data not
shown). In the current study, we used the same doses (50 mg/kg) of curcumin and FLLL12 for comparison purposes. Consistent with our previous study, curcumin failed to inhibit tumor volume at this dose. However, FLLL12 significantly reduced the volume of tumors as compared to vehicle or curcumin-treated groups, although the tumors still grew. Since the tested dose was tolerable without any significant sign of toxicity, future studies should be conducted with higher doses of FLLL12 to achieve more significant results.

It is now well accepted that the induction of apoptosis or other mechanism of cell death is the key to success in tumor elimination (33). Bcl-2 proteins play a crucial role in mitochondria-mediated apoptosis and serve as an excellent target for drug development (34–36). Our findings demonstrate that FLLL12-induced apoptosis is mediated via mitochondrial depolarization, as evidenced by the release of cytochrome C in the cytosol. We also found that FLLL12 treatment modulated the expression of several Bcl-2 proteins, among which the expression of Bcl-2, Bim and Bid favored the induction of apoptosis. We have established their role in mediating FLLL12-induced apoptosis by rescue/overexpression of Bcl-2 and silencing the expression of Bim and Bid. The levels of protection achieved were statistically significant, but not complete when one protein was overexpressed or ablated, suggesting that FLLL12 induced multi-targeted effects. Bid is a pro-apoptotic protein which is truncated by active caspase-8 to tBid, the active form, which facilitates mitochondria-mediated apoptosis (34). The decrease in full length Bid found in our study might be due to a decrease in protein or to truncation of full length Bid to active tBid. However, silencing the expression of Bid significantly protected cells from apoptosis as well as activation of caspase-8 (activates Bid through truncation) by FLLL12, suggesting that the decrease in full length BID is a consequence of the activation of Bid to tBid. Bcl-2 is upregulated in tumors as a target of STAT3 and NF-κB (37–39). Recently, we reported the posttranslational stabilization of Bcl-2 by RRM2 in solid tumors (28). In order to explore the mechanism of inhibition of Bcl-2 by FLLL12, we measured Bcl-2 mRNA which was increased instead of decreased; most likely cells attempted to compensate for the protein loss by enhancing transcription. We also examined whether pretreatment with proteasome inhibitor rescued Bcl-2 protein. However, we failed to rescue the expression of Bcl-2. These data thus suggest that FLLL12 regulates Bcl-2 either at the mRNA stabilization or protein translational level. Finally, once protein synthesis was shut down, FLLL12 had no further effect on Bcl-2 suggesting that the regulation is at the translational level. Indeed, we found strong inhibition of both axes of the mTOR-dependent protein translational pathway by FLLL12.

A protein is the functional molecule that regulates phenotype, and can be modulated at various levels such as replication/copy number change, transcription, translation or posttranslational levels. Upregulation and activation of EGFR occur at a very early stage of SCCHN carcinogenesis. AKT serves as the central hub for many signal transduction pathways regulating tumor initiation and progression, recurrence, drug response and resistance mechanisms (40). AKT is a common downstream target of most oncogenic and growth factor receptor pathways. Similarly, activated AKT can activate numerous downstream signal transduction pathways (41). The recent TCGA reports also suggest that most SCCHN patients has deregulated AKT signaling (42). Small molecule inhibitors as well as monoclonal antibodies targeting the EGFR are currently approved for different malignancies including SCCHN. Multiple drugs targeting the AKT pathways are currently
under different levels of development (43). These drugs are all developed to inhibit the kinase activity of these proteins. However, they can also be targeted at the mRNA level. Our results clearly demonstrate that FLLL12 targets EGFR and AKT at the mRNA level, to ultimately inhibit downstream mTOR and FOXO pathways. We further demonstrate that FLLL12 inhibits Bcl-2 at the translational level. We also found upregulation of Bim, most probably due to activation of FOXO-mediated transcription. In conclusion, our study demonstrates that FLLL12 effectively induces apoptosis and modulates multiple targets. Moreover, the compound is effective in vivo in inhibiting tumor growth and possesses favorable pharmacokinetic features and thus may be an excellent anti-cancer drug. Furthermore, the greater sensitivity of premalignant cells to FLLL12 suggests that this compound might be particularly beneficial in prevention settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Fig. 1.
FLLL12 is more potent than curcumin in inducing apoptosis. (A) Tu686, (B) 886LN, (C) Tu212, (D) UM-22B and (E) MSK-LEUK1 cells were seeded in 6-cm plates to a confluency of 40–50% and treated with the indicated doses of FLLL12 (F) and curcumin (C) for 24 and 48 h. Apoptosis was measured by annexin V-PE staining. Average results of three independent experiments were plotted with standard deviations as error bars. The numbers after F and C indicate the compound doses in µM. Paired two tail student t-test was used to calculate p values. * indicates statistically significant p value (p<0.05). NS means not significant. All comparisons were made with corresponding untreated controls.
Figure 2.
Cleavage of PARP by FLLL12 and curcumin. (A) Tu212, (B) Tu686 and (C) MSK-LEUK1 cells were treated with the indicated doses of FLLL12 and curcumin for 24 h. Whole cell lysates were subjected to Western blotting using PARP antibody that detects both full length and cleaved form of PARP. (D) Release of cytochrome c by FLLL12. Tu212 cells were treated with FLLL12 and curcumin. Cytosolic and mitochondrial fractions were separated and immunoblotted with anti-cytochrome C, Cox4 and ERK antibodies. Data are representative of three independent experiments.
Figure 3.
Role of Bcl-2 proteins in FLLL12-induced apoptosis. (A) Tu212 and Tu686 cells were treated with the indicated doses of FLLL12 and curcumin for 24 h. Expression of Bcl-2, Bcl-xL, Mcl-1, Bid, Bim, PUMA and NOXA were analyzed in whole cell lysates by immunoblotting. (B) Tu686 and Tu686 cells overexpressing Bcl-2 were treated with 2 and 3 µM of FLLL12 for 48 h and apoptosis was measured. Average values from three independent experiments were plotted with standard deviation as error bars. * indicates statistically significant p values. (C) Total RNA from Tu212 and Tu686 were isolated after treatment with FLLL12 and subjected to real-time qPCR for the expression of Bcl-2 mRNA. T1: 1 and 2 µM FLLL12 for Tu212 and Tu686, respectively; T2: 2 and 3 µM FLLL12 for Tu212 and Tu686, respectively; T3: 10 and 15 µM curcumin for Tu212 and Tu686, respectively. NS = not significant. * indicates statistically significant p values.
(p<0.05). All comparisons were made with the corresponding untreated control. (D) The expression of Bim was knocked down in Tu686 cells and apoptosis was measured after 48 h treatment with FLLL12. Average values from triplicate treatments were plotted. (E) The expression of Bid was knocked down in Tu686 cells and apoptosis was measured after 48 h treatment with FLLL12. Average values from triplicate treatments were plotted. All Western blotting data are representative of at least three independent experiments.
Figure 4.
Role of EGFR-AKT signaling in FLLL12-induced apoptosis. (A) Tu212, Tu686 and MSK-LEUK1 cells were treated with different doses of FLLL12 and curcumin. Expression of pEGFR, EGFR, pAKT and AKT were analyzed in whole cell lysates by Western blotting. (B) Vector and CA-AKT transduced Tu686 cells were treated with FLLL12 and curcumin and apoptosis was measured. Average results from three independent experiments were plotted with S.D. as error bars. *, **, *** indicate statistically significant results (p<0.05). (C) Vector and CA-AKT transduced Tu686 cells were treated with FLLL12 and curcumin and the expression of pAKT, AKT, PARP, cleaved caspase 3, Bim and Bcl-2 were analyzed in whole cell lysates. (D) Tu212 and (E) Tu686 cells were treated with the indicated doses of FLLL12 and curcumin and expression of EGFR and AKT mRNA were analyzed by qPCR. Average values of triplicate treatments were presented. (F) Tu212, Tu686 and MSK-LEUK1 cells were treated with different doses of FLLL12 and curcumin. Expression of pmTOR, pS6, S6, p4EBP1 and 4EBP1 were analyzed in whole cell lysates. (G) Tu686 cells were treated with FLLL12 and expression of pFOXO1a and pFOXO3a were analyzed in whole cell lysates. Data are representative of three independent experiments. NS= not significant. * indicates statistically significant p values (p<0.05). All comparisons were made with the corresponding untreated control if not indicated otherwise.
Figure 5.

In vivo efficacy and pharmacokinetic parameters of FLLL12. (A) Mice were administered 200 mg/kg FLLL12 and curcumin by oral gavage. Two or three mice were sacrificed at each time point. Plasma was separated and the concentrations of FLLL12 and curcumin were determined by LC-MS/MS. (B) Pharmacokinetic parameters were determined. (C) Three groups of animals were intra-peritoneally treated with vehicle control, curcumin and FLLL12 (50 mg/kg) as described in “Materials and Methods.” Growth curves were obtained by measuring tumor volume twice a week. FLLL12 significantly inhibited tumor volume when compared with control or curcumin-treated group (p<0.05, Table S1, *comparison between vehicle and FLLL12 treatments; ** comparison among curcumin and FLLL12 treatments). (D) Change in body weight of mice throughout the study.
Table 1

IC₅₀ values of FLLL12 and curcumin at 72 h.

<table>
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<tr>
<th>Cell Line</th>
<th>FLLL-12 (µM)</th>
<th>Curcumin (µM)</th>
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<tbody>
<tr>
<td></td>
<td>IC₅₀</td>
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