TLN-4601, a novel anticancer agent, inhibits Ras signaling post Ras prenylation and before MEK activation

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TLN-4601 is a structurally novel farnesylated dibenzodiazepinone discovered through DECIPHER, Thallion's proprietary drug discovery platform. The compound was shown to have a broad cytotoxic activity (low μmol/l) when tested in the NCI 60 tumor cell line panel and has shown in-vivo antitumor activity in several xenograft models. Related to its farnesylated moiety, the effect of TLN-4601 on Ras mitogen-activated protein kinase signaling was assessed. Downstream Ras signaling events, Raf-1, MEK, and ERK1/2 phosphorylation in MCF7 cells were evaluated by western blot analysis. TLN-4601 prevented epidermal growth factor-induced phosphorylation of Raf-1, MEK, and ERK1/2. This effect was time-dependent and dose-dependent with complete inhibition of protein phosphorylation within 4–6 h at 10 μmol/l. The inhibition of Ras signaling was not mediated by the inhibition of protein prenylation, documented by the lack of effect TLN-4601 on the prenylation of HDJ2 (specific substrate of farnesyltransferase), RAP1A (specific substrate of geranylgeranyl transferase-1), or Ras. As TLN-4601 did not inhibit EGFR, Raf-1, MEK or ERK1/2 kinase activities, the inhibitory effect of TLN-4601 on Ras signaling is not mediated by direct kinase inhibition. Using an Elk-1 trans-activation reporter assay, we found that TLN-4601 inhibits the MEK/ERK pathway at the level of Raf-1. Interestingly, TLN-4601 induces Raf-1 proteasomal-dependent degradation. These data indicate that TLN-4601 may inhibit the Ras-mitogen-activated protein kinase-signaling pathway by depleting the Raf-1 protein. *Anti-Cancer Drugs* 21:543–552 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

DECIPHER, the drug discovery platform of Thallion Pharmaceuticals Inc., can predict chemical structures of potential new drugs by evaluating gene sequences and genetic loci obtained from microorganisms [1–3]. TLN-4601 (formerly ECO-4601), a small molecular weight farnesylated dibenzodiazepinone, is one of the compounds that was discovered using this technology [4,5]. In-vitro cytotoxicity and in-vivo efficacy studies, together with safety data and early signs of antitumor activity observed in phase I clinical testing, suggest that TLN-4601 is a good candidate for additional clinical studies assessing its benefits against brain and other solid tumors [6].

Our earlier data indicated that TLN-4601 specifically binds the peripheral benzodiazepine receptor (PBR) [6]. Although the exact function of the PBR (more recently known as the translocator protein [7]) remains a matter of debate, it has been implicated in heme and steroid synthesis, cellular growth and differentiation, and oxygen consumption and apoptosis (reviewed in [8,9]). PBR ligands have been reported to inhibit cell proliferation and induce apoptosis. In contrast, no clear antitumor activity resulting from their direct interaction with PBR *per se* has been documented [10–13]. Recently, we have shown that Jurkat cells, naturally devoid of PBR expression, are as sensitive towards TLN-4601 as their PBR-transfected counterpart, Jurkat-PBR (Bertomeu *et al.*, submitted). These data imply that other cellular targets likely contribute to the broad antitumor activity of TLN-4601.

Related to its farnesylated moiety, the effect of TLN-4601 on the Ras mitogen-activated protein kinase (MAPK) signaling pathway was assessed. The Ras-MAPK signaling pathway is a well-validated oncogenic pathway based on its central role in regulating the growth and survival of cells from a broad spectrum of human tumors [14]. Furthermore, Ras is the most frequently mutated oncogene, occurring in approximately 30% of all human cancers. As a cellular system, we used human breast MCF7 cells as they express wild-type Ras proteins and the Ras signaling pathway is inducible by epidermal growth factor (EGF). We show here that TLN-4601 inhibits the Ras-MAPK signaling pathway, after stimulation with EGF or serum. We verified whether TLN-4601 interfered with Ras processing by monitoring FPTase and GGPTase I activities. No mobility shifts of either HDJ2 or Rap1A (surrogate markers of FPTase and GGPTase I, respectively) were observed in cells exposed to TLN-4601 for up to 48 h. In contrast, a strong inhibition of EGF-induced phosphorylation of Raf-1, MEK, and ERK1/2

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was observed. To further elucidate the site of action of TLN-4601 within the intracellular signaling cascade, we used a reporter assay to measure the transcriptional activity of ELK-1, a downstream target of ERK. Our data indicate that TLN-4601 inhibits Ras-MAPK signaling upstream of MEK.

Materials and methods

Chemicals and cell cultures

TLN-4601, obtained by fermentation as described earlier [4,5], was prepared as a 100 mmol/l dimethyl sulfoxide (DMSO) stock and aliquots were stored frozen at -20°C. At the time of testing, the aliquots were thawed and the stock solution was further diluted in cell culture media containing 10% or 0.1% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, Maryland, USA). The molecular structure of TLN-4601 is shown in Fig. 1a. Geldanamycin (GA) and MG132 were purchased from Tocris Bioscience (Ellisville, Missouri, USA); lovastatin and UO126 were purchased from Sigma-Aldrich (St Louis, Missouri, USA), 17-allyaminogeldanamycin was obtained from BIOMOL International LP (Plymouth Meeting, Pennsylvania, USA) and radicicol from StressMarq Biosciences Inc., (Victoria, British Columbia, Canada). All drugs were diluted in DMSO.

The human breast MCF7 tumor cell line was purchased from the American Type Culture Collection (Manassas, Virginia, USA). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS and maintained in a humidified atmosphere at 37°C with 5% CO_2. Cell lines started from frozen stocks were maintained in culture for 15–20 passages and were free of *Mycoplasma* (routinely tested by PCR; Sigma-Aldrich).

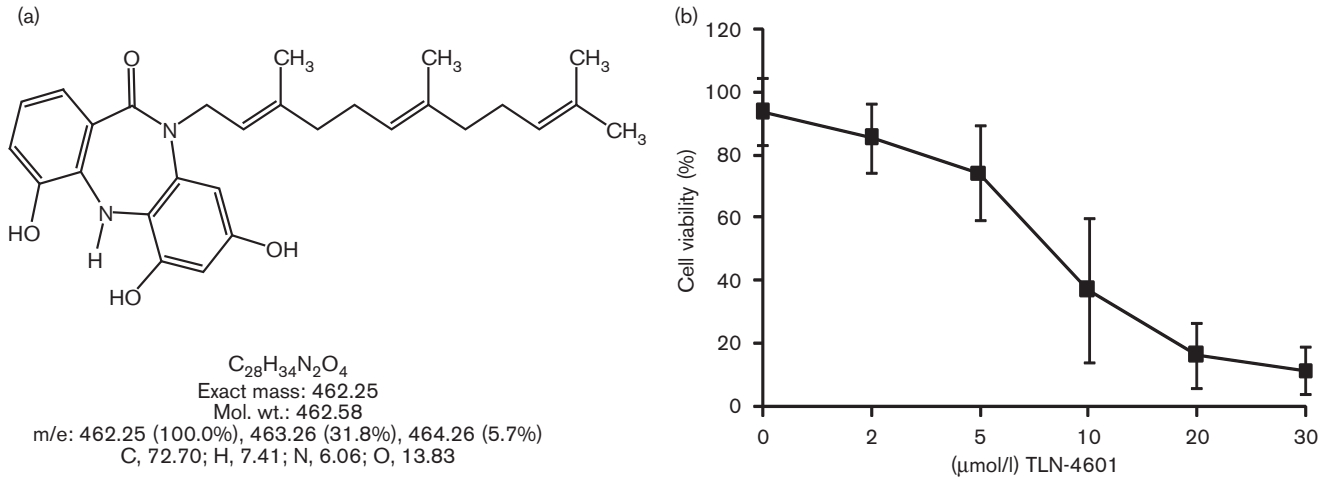
Cell viability assays

Exponentially growing cells were seeded in 96-well plates at a density of 2.5×10^4 cells per well (cell numbers were determined using a haemocytometer) and allowed to attach overnight. TLN-4601 was added at 0, 2, 5, 10, 20, and 30 μmol/l, respectively, in a final concentration of RPMI-0.1% FBS and 0.05% DMSO. Cells were further incubated for 24 h and subsequently exposed to 100 ng/ml EGF for 5 min at 37°C. Cell viability was assessed with the CellTiter-Glo assay (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The experiment was performed in triplicate and repeated three times.

Analysis of Ras-MAPK signaling pathway

To study the effect of TLN-4601 on the Ras-MAPK signaling pathway, exponentially growing cells were seeded onto 60-mm tissue culture dishes ($\sim 0.8 \times 10^6$ cells per dish) for 24 h. For the time-dependent inhibition response, the cells were treated with a fixed concentration (10 μmol/l) of TLN-4601 in the culture medium supplemented with 0.1% FBS for 30 min, 1, 4, and 6 h, respectively, and subsequently exposed to EGF at 100 ng/ml for 10 min at 37°C. The dose-dependent inhibition response was evaluated after treating cells to increasing concentrations of TLN-4601 overnight, in 0.1% FBS followed by the 10 min EGF stimulation. Control plates consisted of cells incubated in culture medium containing 0.1% FBS and 0.05% DMSO (vehicle), with or without EGF stimulation. At the end of each treatment, the medium was removed and cells rinsed with ice-cold PBS. The cells were harvested by scraping and cell pellets were lysed in ice-cold RIPA

Fig. 1



(a) Molecular structure of TLN-4601. TLN-4601 is expressed in the fermentation broth of an actinomycete (*Micromonospora* sp.) and purified by a series of chromatographic methods as described earlier [5]. (b) Cell survival after TLN-4601 treatment. Epidermal growth factor-induced MCF7 cell survival was assayed using the CellTiter-Glo Luminescent Cell Viability Assay. Data are expressed as treated over control (dimethyl sulfoxide), and error bars represent the SD of three independent experiments.

buffer [(50 mmol/l Tris–HCl pH 7.4, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mmol/l NaCl, and 1 mmol/l EDTA) containing protease (Roche Applied Science, Mannheim, Germany) and phosphatase (EMD4 Biosciences, Calbiochem, San Diego, California, USA) inhibitors] for 20 min on ice. Insoluble material was pelleted after centrifugation at $12\,000 \times g$ and discarded. The protein concentration of each lysate was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA). Equivalent amounts of protein (20–30 μg) were separated on 10% SDS–PAGE under reducing conditions, and transferred onto nitrocellulose membranes (0.2 μm; Bio-Rad Laboratories). Membranes were blocked with 5% skim milk in 1 × Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature, and incubated overnight at 4°C with the following primary antibodies: phospho-Raf-1 (Ser338), Raf-1, phospho-MEK1/2 (Ser217/221), phospho-p44/42 (Thr202/Tyr204, p-ERK1/2), and p44/42 (ERK1/2) MAP kinases (all from Cell Signaling Technology Inc., Boston, Massachusetts, USA) and GAPDH (SantaCruz Biotechnology Inc., Santa Monica, California, USA). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno-Research Laboratories Inc., West Grove, Pennsylvania, USA) for 1 h at room temperature in 0.5% skim milk-Tween-20, visualized by treating the membranes with enhanced chemiluminescence reagents (Millipore, Mississauga, Ontario, Canada) and exposing them to BioMax Light Films (Kodak, Mississauga, Ontario, Canada). The resulting radiograph films were scanned and digitized using the UN-SCAN-IT gel software (Silk Scientific Corporation, Orem, Utah, USA).

Kinase activity assays

The effect of TLN-4601 on human EGF receptor (EGFR), RAF-1, MEK1, MAPK1 (ERK1), and MAPK2 (ERK2) kinase activity was evaluated at Upstate Ltd, Millipore (Upstate Kinase Profiler Service, Dundee, UK). TLN-4601 was tested at 0.5 and 5 μmol/l in a final volume of 25 μl according to standard protocols developed by Upstate Ltd. Briefly, purified recombinant human enzymes were incubated with 25 mmol/l Tris pH 7.5 containing EGTA, a specific substrate, and γ-^{32}P-ATP. The reaction was initiated with the Mg-ATP mix (ATP concentration and positive control depended on the kinase evaluated) and incubated for 40 min at room temperature. The reaction was stopped by the addition of 5 μl of a 3% phosphoric acid solution; aliquots were spotted on filters and counted. Detailed procedures are available on the Millipore Upstate website.

Analysis of protein prenylation

To study the effect of TLN-4601 on protein prenylation, exponentially growing cells were seeded in 60-mm cell culture dishes at a density of 2.5×10^6 cells per dish in RPMI containing 10% FBS. After the 18 h incubation period, TLN-4601 or lovastatin was added at a final concentration of 3, 10, and 30 μmol/l for 24 h. Cells were also treated with the vehicle alone (0.05% DMSO) for the same incubation period. Treated and control cells were processed as described above. Equal amount of proteins (30 μg) were separated by SDS-PAGE (8% for HDJ2 and 20% for Ras and RAP1A) and transferred onto nitrocellulose membranes and probed as described above with the following primary antibodies: HDJ2 (GeneTex Inc., San Antonio, Texas, USA), Pan-Ras (EMD4 Biosciences), RAP1A and GAPDH (Santa Cruz Biotechnology Inc.).

Luciferase assay

The PathDetect Elk-1 trans-reporting system was purchased from Stratagene (La Jolla, California, USA). pCMV-H-Ras V12, pCMV-Raf CAAX and pCMVβ were from Clontech (Mountain View, California, USA). pCDNA3-Raf BXB was kindly provided by Dr Adrienne D. Cox (University of North Carolina, Chapel Hill, North Carolina, USA). Trans-activator (GAL4-dbd Elk-1 fusion protein) plasmid construct (pFA2-Elk1; 0.2 μg) was cotransfected with a reporter plasmid (pFR-Luc GAL4 UAS-luciferase; 0.1 μg) and 0.1 μg of constructs expressing various constitutively active components of the Ras-MAPK pathway (H-Ras V12, Raf-1 CAAX, Raf-1 BXB or MEK (S218222E, Δ32-51). Twenty-four hours after transfection, MCF7 cells were treated for 18 h with increasing concentrations of TLN-4601 or with 10 μmol/l U0126 (a MEK1/2 inhibitor). Cells were harvested and luciferase activity was determined using a commercial Luciferase Assay (Promega) according to the manufacturer's instructions. The experiment was carried out in duplicate and repeated at least three times. Results were corrected for background luciferase activity using pFC2-dbd (expressing only the GAL4 DNA binding domain) and normalized with a cotransfected pCMVβ plasmid, expressing β-galactosidase. Data are expressed as treated (TLN-4601, UO126) over control (Elk-1 activity without compounds). Values represent mean ± SD of three independent experiments.

Raf-1 degradation analysis

MCF7 cells growing in 0.1% FBS-RPMI were treated with 1, 4, 10, and 20 μmol/l of the proteosome inhibitor MG132 for 1 h followed by the addition of TLN-4601 at 10 μmol/l for 18 h. At the end of the treatment period, cells were lysed and analyzed for Raf-1 expression by western blot analysis as described above. In addition, MCF7 cells were seeded in 6-well plates at 1×10^5 cells per well in RPMI supplemented with 10% FBS and incubated at 37°C overnight. Cells were transfected with 4 μg of DNA of pCW8 (expressing Myc-tagged ubiquitin K48R mutant protein) or control vector (empty vector) using a FuGENE HD transfection reagent (Roche Applied Science). Forty-eight hours after transfection, cells were treated with 30 μmol/l TLN-4601 or 2 μmol/l GA, respectively, for 18 h. The cells were harvested and

Raf-1 levels were determined by western blot analysis. The pCW8 plasmid was a generous gift from Dr R. Kopito (Stanford University School of Medicine, Stanford, California, USA).

Analysis of Hsp90 client proteins

Exponentially growing MCF7 cells (10% FBS-RPMI) were treated with increasing concentrations of TLN-4601 for 18 h, as indicated. At the end of the treatment period, cells were lysed as described above and analyzed for a selected panel of Hsp90 client proteins using specific antibodies: Raf-1, AKT, Hsp90, Hsp70 (Cell Signaling Technology Inc.), and HER2/Neu (Santa Cruz Biotechnology Inc.).

Hsp90 binding assay

Hsp90 binding assays were performed as described earlier by Kamal *et al.* [15]. MCF7 cells were lysed in lysis buffer (20 mmol/l HEPES, pH 7.3, 1 mmol/l EDTA, 5 mmol/l $MgCl_2$, 100 mmol/l KCl). Cellular extracts (1 mg of total protein) were incubated with increasing concentrations of TLN-4601 for 30 min at 4°C, followed by 1 h incubation with biotin-GA (Biotin GA; BioMol, Plymouth Meeting) linked to dynabeads M-280 streptavidin (Invitrogen). The beads were separated from the supernatant using a magnetic rack, washed three times in lysis buffer and boiled in SDS-PAGE sample buffer for 5 min. The samples were separated on an SDS-PAGE gel and western blot analysis was performed with an anti-HSP90 antibody (Cell signaling Technology Inc.).

Results

TLN-4601 cytotoxic activity

The effect of TLN-4601 on EGF-induced MCF7 cells was assessed using the CellTiter-Glo Luminescent Cell Viability Assay. The assay determined the number of viable, metabolically active cells in culture based on the quantification of total cellular ATP content. The calculated IC_{50} (drug concentration resulting in 50% reduction in ATP levels) is approximately 7.5 μmol/l (Fig. 1b).

TLN-4601 inhibits the Ras-MAPK pathway

We examined the effect of TLN-4601 on the Ras-MAPK signaling pathway by monitoring the phosphorylation levels of Raf-1, MEK, and ERK1/2 by western blot analysis. In low serum conditions and in the absence of EGF, levels of p-Raf (Ser338) are negligible and those of p-MEK and p-ERK1/2 are undetectable (Fig. 2a, lane 1). EGF stimulation results in a strong induction of pRaf-1, pMEK, and pERK1/2 phosphorylation levels (Fig. 2a, lane 2). EGF-induced phosphorylation of Raf-1, MEK, and ERK is significantly decreased by preincubating cells with TLN-4601, and is observed within 4 h of treatment (Fig. 2a). We also noted that TLN-4601 causes a decrease in the amount of total Raf-1 protein, which seems to coincide with decrease in phosphorylation. The dose–response relationship was evaluated in MCF7 cells after an overnight incubation with TLN-4601. A decrease in pERK1/2 occurred between 10 and 30 μmol/l whereas a decrease in pRaf-1 levels and Raf-1 total protein levels was observed between 1 and 3 μmol/l (Fig. 2b).

Effect of TLN-4601 on kinase activity

To further understand the mechanism by which TLN-4601 inhibits Ras-MAPK signaling, we assessed whether the compound inhibited kinase activity directly. An enzymatic binding assay was carried out between TLN-4601 and the EGFR, Raf-1, MEK1, ERK1, and ERK2. For this direct binding assay, TLN-4601 was tested at 0.5 and 5 μmol/l. Evaluation of TLN-4601 on the selected kinases was performed at Upstate Millipore using their kinase Profiler radiometric screening assay. Results obtained from Upstate Millipore and summarized in Table 1 indicate that TLN-4601 does not inhibit EGFR, Raf-1, MEK1, ERK1, or ERK2 kinase activities.

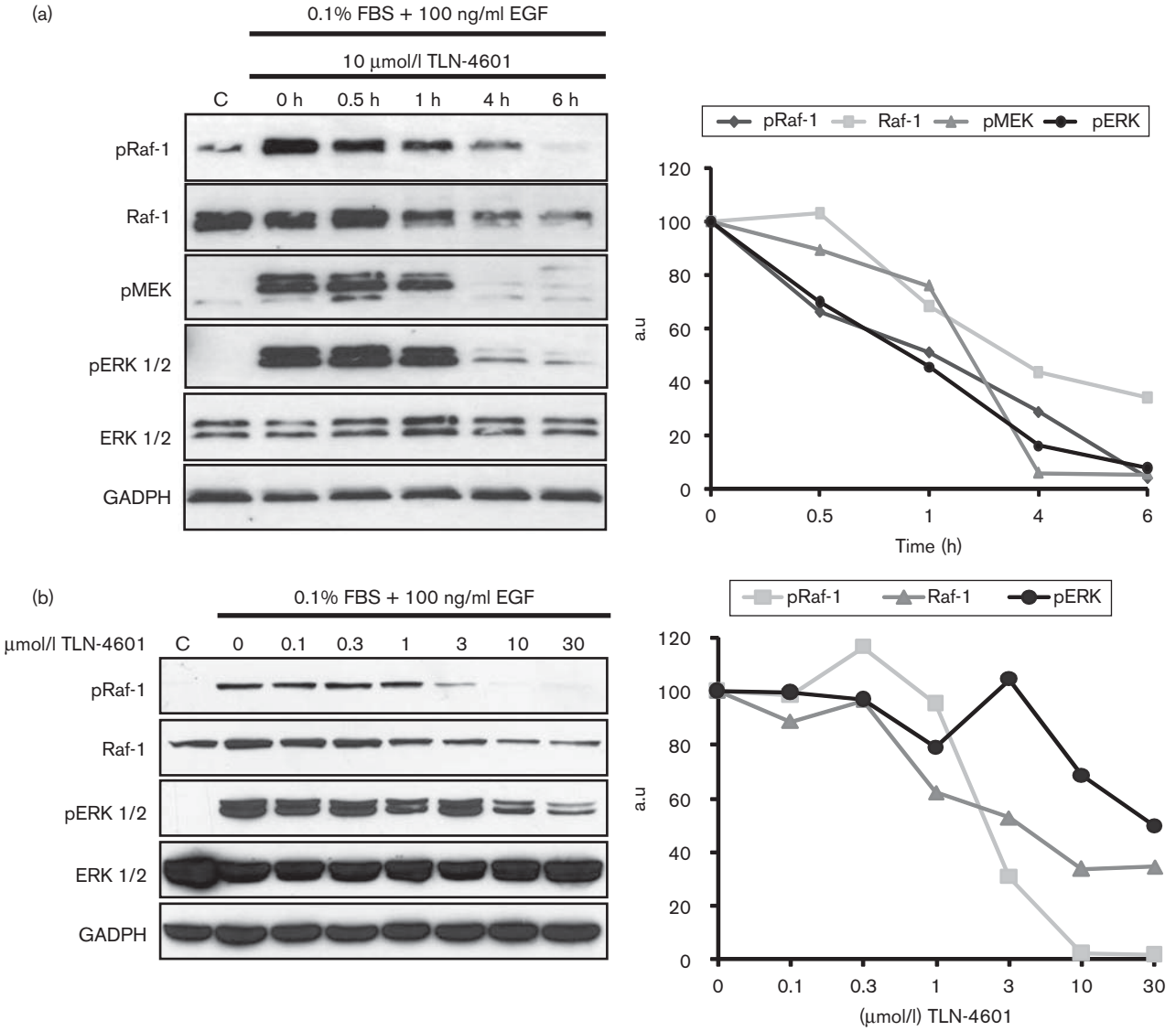
Effect of TLN-4601 on protein prenylation

To determine whether TLN-4601 is an inhibitor of farnesyltransferase (FTase) and/or geranylgeranyl transferase-1 (GGTase-1), western blot analysis was performed to monitor prenylation inhibition of HDJ2, a protein prenylated exclusively by FTase [16,17], and RAP-1A, a protein prenylated exclusively by GGTase-I [18]. Unprenylated (U) and prenylated (P) proteins can be distinguished by their different electrophoretic mobilities; the unprenylated forms of HDJ2 and RAP-1A displayed reduced mobility in SDS-PAGE relative to their prenylated forms. As a positive control and to identify both forms, we used lovastatin, which inhibits HMG-CoA reductase and blocks the biosynthesis of intermediates required for protein prenylation such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate [19]. No mobility shift is observed in either HDJ2 or RAP-1A when MCF7 cells are exposed to increasing concentrations of TLN-4601 for 24 h (Fig. 3). As expected, lovastatin treatment results in a dose-dependent inhibition of HDJ2 and RAP1A processing, as indicated by the mobility shift observed with increasing concentrations of drug. Ras protein processing was also evaluated using a pan-Ras antibody and, as shown in Fig. 3, whereas lovastatin interfered with Ras processing TLN-4601 did not. This result also shows that TLN-4601 does not affect Ras protein levels.

TLN-4601 inhibits Ras-MAPK signaling downstream of Ras, but upstream of MEK

To explore how TLN-4601 inhibits ERK activation, we performed a reporter assay that measures the transcriptional activity of Elk-1, a nuclear target of ERK. Transactivator (GAL4-Elk1 fusion protein) and reporter (GAL4 UAS-luciferase) plasmids were cotransfected with various active components of the Ras-MAPK pathway. They included H-Ras-V12, Raf-1 BXB, which only contains

Fig. 2



TLN-4601 inhibits the Ras-MAPK signaling pathway. (a) Time-dependent inhibition of the Ras MAPK signaling pathway. Exponentially growing MCF7 cells were treated with 10 μmol/l TLN-4601 in RPMI supplemented with 0.1% FBS for 0.5, 1, 4, and 6 h. At the end of the different drug-incubation periods, cells were exposed to epidermal growth factor (EGF, 100 ng/ml) for 10 min. Control plates consisted of cells incubated in RPMI supplemented with 0.1% fetal bovine serum (FBS) and 0.05% dimethyl sulfoxide (vehicle), with or without EGF stimulation. (b) Dose-dependent inhibition of Ras-MAPK signaling. Exponentially growing MCF7 cells were serum starved for 9 h in 0.1% FBS-RPMI and then treated with 1, 3, 10, and 30 μmol/l TLN-4601 for 18 h. At the end of the drug-incubation periods, cells were exposed to EGF (100 ng/ml) for 5 min. Cellular extracts (30 μg protein) were separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were sequentially probed with pRaf-1 (Ser 338), Raf-1, pERK1/2 (phospho-p44/42 MAP kinase Thr202/Tyr204), ERK1/2 (p44/42 MAP kinase), and GADPH (loading control). Graphs on the right provide a densitometric quantification of the western blots (a.u. denotes arbitrary units). Blots are representatives of three separate experiments.

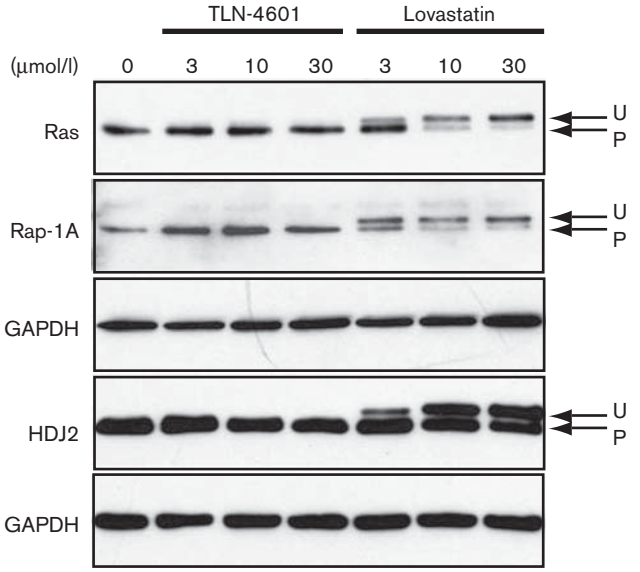
the kinase domain of Raf-1, Raf-1 CAAX, which is constitutively targeted to the plasma membrane, and activated MEK1 (S218222E, Δ32-51). Phosphorylation of the transcription activation domain of GAL4 Elk-1 by ERK at the end of the MAPK signaling cascade activates transcription of the luciferease gene from the reporter plasmid. Transfections with each of these proteins stimulated endogenous ERK phosphorylation as documented by western blot analysis (Fig. 4a), and resulted in Elk-1-mediated transcriptional response as measured by an increase in luciferase activity (Fig. 4b). UO126, a commercial MEK inhibitor, was used as a positive control to inhibit the resulting Elk-1 activation. TLN-4601 inhibits Elk-1 trans-activation by constitutively activated H-Ras (V12) and Raf-1 BXB, but not by membrane bound Raf-1 CAAX or MEK. This observation indicates that TLN-4601 inhibits ERK signaling downstream or at the level of Raf-1 and upstream of MEK.

Table 1 Effect of TLN-4601 on the kinase activity of EGFR, Raf-1, MEK1, ERK1, and ERK2

	Kinase activity (%) ± SD	
Kinases	TLN-4601 (0.5 μmol/l)	TLN-4601 (5 μmol/l)
EGFR	128 ± 6	127 ± 9
Raf-1	114 ± 12	94 ± 2
MEK1	106 ± 2	98 ± 1
MAPK1 (ERK1)	97 ± 2	73 ± 3
MAPK2 (ERK2)	116 ± 1	110 ± 1

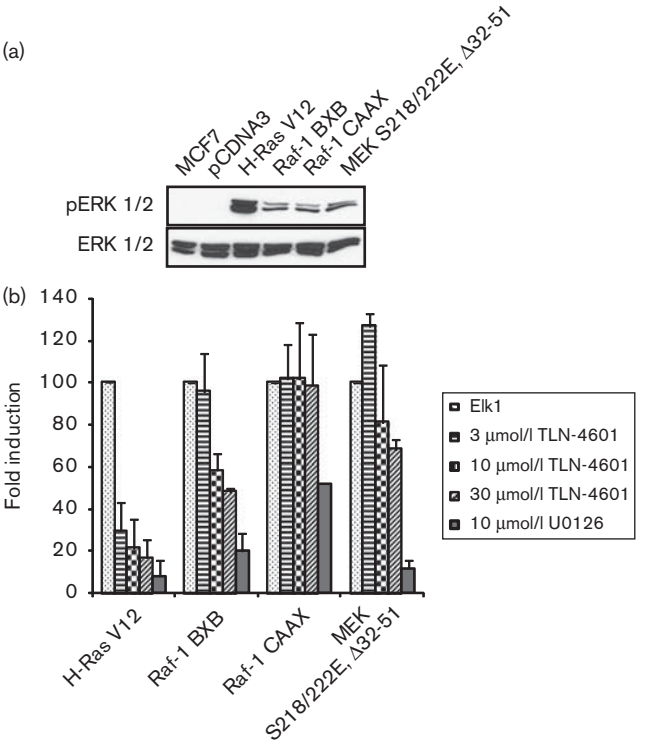
Data are expressed as the percentage of enzyme activity in the presence of TLN-4601 over positive control. The experiment was carried out at Upstate Millipore. Results are the mean of three independent experiments ± SD.
EGFR, epidermal growth factor receptor.

Fig. 3



TLN-4601 does not inhibit protein prenylation. MCF7 cells were treated with 3, 10, and 30 μmol/l TLN-4601 or lovastatin for 24 h. The control was treated with vehicle (0.05% dimethyl sulfoxide) for 24 h. Cellular lysates were prepared and western blot analysis was performed as described in Materials and methods section. Prenylated (P) and unprenylated (U) forms for each protein are indicated. GAPDH was used as a loading control. The blot is representative of three independent experiments.

Fig. 4

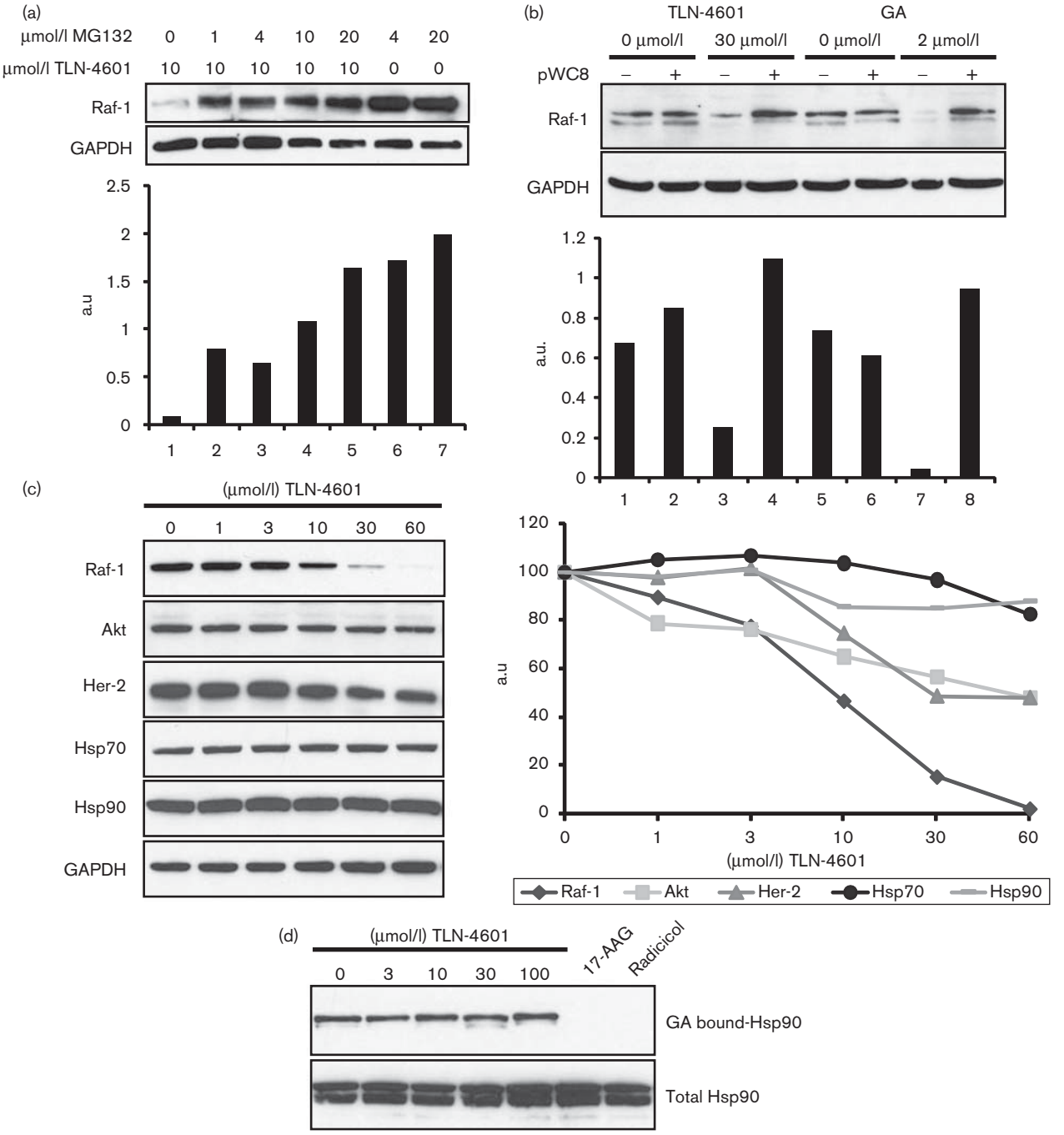


TLN-4601 inhibits Ras-MAPK signaling downstream of Ras, but upstream of MEK. (a) ERK phosphorylation by different constitutively active members of the MAPK signaling pathway. MCF7 cells were transfected with pCDNA3, pCMV-H-Ras V12, pCDNA-Raf BXB, pCMV-Raf CAAX or pFC-MEK1. Twenty-four hours after transfection cells were harvested and cell lysates were analyzed by western blot. (b) Effect of TLN-4601 on Elk-1 trans-activation. MCF7 cells were transiently co-transfected with the fusion *trans*-activator plasmid (pF2A-Elk1; GAL4 dbd-Elk1 fusion protein), the reporter plasmid (pFR-Luc; GAL4 UAS-luciferase) and expression vectors for constitutively active H-Ras V12, Raf-BXB, Raf-CAAX or MEK1 (S281/222E, Δ32-51). TLN-4601 was added 24 h after transfection and luciferase activity monitored 18 h after treatment. Results were corrected for background luciferase activity using pFC2-dbd (expressing only the GAL4 DNA binding domain) and normalized with a cotransfected pCMVβ plasmid, expressing β-galactosidase. Data are expressed as treated (TLN-4601, UO126) over control (Elk-1 activity without compounds). Values represent mean ± SD of three independent experiments.

TLN-4601 induces Raf-1 degradation through proteasome pathway

Raf-1 stability and function depend on its association with Hsp90 [20–23]. It has been shown that disturbing the Hsp90/Raf-1 complex results in Raf-1 ubiquitination, targeting it to the proteasome, and leading to Raf-1 protein degradation [24,25]. To determine whether TLN-4601-induced Raf-1 degradation is mediated by the proteasome, we tested whether the proteasome inhibitor, MG132, could prevent Raf-1 decrease. These studies were carried out in normal serum conditions (10%), as we were not evaluating cell signaling. In these conditions, a dose–response analysis indicates that 10 μmol/l of TLN-4601 produces a significant decrease in Raf-1 protein levels (data not shown). MCF7 cells were thus pretreated with increasing concentrations of MG132 (as indicated) for 1 h followed by overnight treatment with 10 μmol/l of TLN-4601. As shown in Fig. 5a (lanes 2 to 4), MG132 prevents the Raf-1 protein decreases observed after treatment with 10 μmol/l of TLN-4601 (Fig. 5a, lane 1). These data were further supported by other experiments using a plasmid (pWC8) encoding a ubiquitin mutant (K48R) protein, which prevents the formation of polyubiquitin chains and the subsequent protein degradation by the proteasome. Neither TLN-4601 nor GA, an Hsp90 inhibitor (used as a control), can induce Raf-1 degradation in MCF7 cells that express the K48R mutant version of ubiquitin (Fig. 5b, lanes 4 and 8).

Fig. 5



TLN-4601 induces proteasomal degradation of Raf-1. (a) MCF7 cells growing in 10% serum were treated for 1 h with increasing concentrations of the proteasome inhibitor (MG132) followed by 18 h treatment with 10 µmol/l TLN-4601. Cellular extracts were prepared as described in Materials and methods section. Raf-1 levels were analyzed on a 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were sequentially probed with Raf-1 and GADPH (loading control). (b) MCF7 cells were transfected with a plasmid expressing the ubiquitin mutant K48R protein (pWC8; +) or its empty vector (–) and then treated with TLN-4601 (30 µmol/l) or GA (2 µmol/l). Cell lysates were subjected to SDS/PAGE followed by western blot analysis with antibodies against Raf-1 and GAPDH. (c) Effect of TLN-4601 on the expression of Hsp90 client proteins. 24 h after plating, exponentially growing MCF7 cells were treated with 1, 3, 10, 30, and 60 µmol/l TLN-4601 for 18 h, in normal serum conditions (10%). Cellular extracts (30 µg) were used to detect Raf-1, Akt, Her-2, Hsp70, Hsp90, and GADPH (loading control) by western blot analysis. (d) TLN-4601 is not a classical Hsp90 inhibitor. Cell lysates from MCF7 were incubated with increasing concentrations of TLN-4601, 1 µmol/l 17-AAG or 1 µmol/l radicicol for 30 min at 4°C, and then incubated for 1 h with biotin–geldanamycin linked to BioMag streptavidin magnetic beads. Hsp90 complexed with biotin–geldanamycin magnetic beads and total cellular Hsp90 were analyzed by western blot using specific anti-Hsp90 antibody. Graphs provide a densitometric quantification of the western blots (a.u. denotes arbitrary units). Blots are representatives of three separate experiments.

Effect of TLN-4601 on Hsp90 client proteins

As Raf-1 is an Hsp90 client protein, we assessed whether TLN-4601 affected other Hsp90 client proteins. MCF7 cells growing in normal serum conditions (10%) were treated with increasing concentrations of TLN-4601 and the levels of Hsp90 client proteins, AKT, Raf-1, and HER-2/Neu as well as Hsp90 and Hsp70 were assessed by western blot hybridization. Although TLN-4601 treatment results in a significant dose-dependent decrease in Raf-1 protein levels, it has a modest effect on HER-2 and AKT protein levels. Moreover, TLN-4601 does not increase Hsp70 protein levels (Fig. 5c), the latter being a hallmark of Hsp90 inhibition [26].

TLN-4601 is not a classical Hsp90 inhibitor

To investigate whether TLN-4601 binds Hsp90, a competitive binding assay using biotin-GA was performed. MCF7 cell lysates were incubated with increasing concentrations of TLN-4601 and endogenous Hsp90 was captured by further incubating TLN-4601 treated cell lysates with biotin-GA. Although 17-allyaminogeldanamycin and radicicol, two well-known Hsp90 inhibitors, were able to displace biotin-GA from Hsp90 TLN-4601 had no effect (Fig. 5d). These data indicate that TLN-4601 does not bind to the conserved binding pocket domain of Hsp90 suggesting a different mechanism of action.

Discussion

This study presents preliminary data on the mechanism of action of TLN-4601, a natural farnesylated dibenzodiazepinone compound, presently in a phase II clinical trial for the treatment of glioblastoma multiforme. TLN-4601 is one of the natural compounds identified using Thallion's proprietary DECIPHER platform [4,5], a technology that combines genomics and bioinformatics to discover novel chemical entities from microorganisms [1–3]. As the potential activity of the compound was first identified using a functional screen, the mechanism by which it inhibited cell growth was unknown. As TLN-4601 contains a farnesyl side chain, we were interested in determining whether the molecule could act as a functional Ras antagonist. Numerous approaches to inhibit Ras function have been explored, including the farnesyltransferase inhibitors (FTIs), which inhibit Ras prenylation and S-trans, trans-farnesylthiosalicylic acid (FTS), which dislodges Ras from the membrane [27]. Therefore, we investigated whether TLN-4601 might act as well-known Ras inhibitors.

To assess whether TLN-4601 affects the Ras-MAPK signaling cascade, we chose the human breast cancer MCF7 cell line as a model experimental system for the following reasons: (i) MCF7 cells express wild-type Ras proteins and (ii) the Ras signaling pathway is inducible by EGF. TLN-4601 inhibits EGF-induced phosphorylation of Raf-1, MEK, and ERK1/2 proteins. The effect is dose and time dependent, occurring between 3 and 10 μmol/l and within 4 h. We also noted a significant decrease in total Raf-1 protein levels. Although the decrease in pRaf-1 appears to coincide with the decrease in Raf-1 protein levels, we need to note that only the serine 338 phosphorylated levels were evaluated, which may not be representative of full Raf-1 kinase activity [28]. Furthermore, a small decrease in total protein levels could result in a relatively higher measurable decrease in phosphorylation levels. To understand how and at what level(s) Ras-MAPK signaling is inhibited, the effect of TLN-4601 on different steps of the pathway was evaluated.

The association of Ras proteins with the inner surface of the plasma membrane is required for Ras signaling and transforming activity. To properly associate with the cell membrane, Ras must first undergo prenylation [29]. The addition of a 15-carbon farnesyl isoprenoid to a cysteine near the COOH terminus of newly synthesized Ras is carried out by the enzyme Ftase. Consequently, efforts have been made on the design of FTIs [30–32]. Early investigations have indicated that FTIs induce antitumor activity *in vitro* and *in vivo* [33–37]. However, while the H-Ras isoform is sensitive to FTIs, N-Ras and K-Ras isoforms, associated with the majority of mutations in human cancer, escape FTI inhibitory effects by being alternatively modified by the addition of a 20-carbon geranylgeranyl group on the same cysteine residue by the enzyme GGTase-type I [29]. Our results indicate that TLN-4601 does not affect protein prenylation and hence is not a farnesyl and/or GGTase inhibitor.

Another class of functional Ras antagonists are the synthetic S-prenyl derivatives of rigid carboxylic acids, the most characterized being FTS [27,38,39]. Interestingly, FTS (Salirasib; Concordia Pharmaceuticals, Ft. Lauderdale, Florida, USA) and TLN-4601 share the same farnesylated side chain. It is thought that FTS specifically interacts with Ras farnesylcysteine binding domains and affects membrane anchorage of Ras. In addition, FTS-induced Ras dislodgement from the membrane renders the protein more susceptible to degradation [40,41]. On account of the structural similarity between FTS and TLN-4601, it was tempting to speculate that TLN-4601 may work in a similar way. However, our results show that TLN-4601 did not affect total Ras protein levels.

To determine at which level TLN-4601 inhibited Ras-MAPK signaling, a reporter assay that measures the transcriptional activity of Elk-1, a downstream target of ERK, was used. TLN-4601 inhibits Elk-1 trans-activation by constitutively activated H-Ras (V12) and cytosolic Raf-1 BXB, but not by membrane-bound Raf-1 CAAX or activated MEK. This assay allowed us to further refine our understanding of the TLN-4601 mechanism of action by determining the level at which TLN-4601 exerts its inhibitory effects on the MAPK signaling cascade.

TLN-4601 inhibits the signaling cascade downstream of Ras, but upstream of MEK. This leaves the Raf-1 protein as a potential candidate and suggests that TLN-4601-induced reduction in Raf-1 levels might be the way used by TLN-4601 to attenuate ERK signaling. A possible reason for the observed difference in functional inhibition between the Raf-1 BXB and Raf-1 CAAX experiments is likely because of the CAAX motif introduced to the Raf-1 protein. The C-terminal CAAX motif is a signature for carboxyl methylation, a post-transcriptional modification that has numerous functions: protein activation, membrane attachment and protection from proteolytic degradation [42]. Adding a C-terminal prenylcysteine (CAAX) to Raf-1 protein allows its artificial anchorage to the membrane, and therefore, mimics its translocation to the membrane and activation. Moreover, it could protect Raf-1 from degradation after TLN-4601 treatment. Raf-1 BXB, localized more diffusely throughout the cytoplasm, may be more easily degraded after TLN-4601 treatment.

We observe that TLN-4601 induces Raf-1 degradation. Raf-1 protein is part of a large molecular complex that contains Hsp90, p50 and other cellular proteins [23]. Hsp90 functions as a chaperone in this complex, maintaining stability of Raf-1 and protecting it from proteasomal degradation. Treatment of cells with TLN-4601 promotes the proteasomal degradation of Raf-1. These findings suggest that TLN-4601 may disturb the Raf-Hsp90 complex. Hsp90 inhibitors such as GA and radicicol lead to Raf-1 degradation and inhibit the MAPK signaling pathway [22,24,25,43]. These drugs interact with the ATP binding site of Hsp90 and favor an Hsp90 conformation that triggers assembly of the destabilizing and proteasome-targeting chaperone machinery [44–47]. We compared the TLN-4601 structure to Hsp90 inhibitors and found no similarity, suggesting that TLN-4601 could have a different mode of action. This hypothesis is supported by the observation that TLN-4601 did not induce Hsp70 expression as shown in published reports with GA [26,48,49] and does not compete for the ATP site with biotin-GA (biotin-GA displacement assay). TLN-4601 may induce Raf-1 degradation by disturbing Raf-1 interaction with one of the other components of the Hsp90 stabilizing complex, by acetylating Hsp90, which has been shown to inhibit ATP binding and therefore, preventing its association with client proteins [50,51] or more likely, by interacting directly with Raf-1. These hypotheses are currently under investigation.

Our data suggest that TLN-4601 acts differently from FTIs and FTS, and inhibits Ras signaling downstream or at the level of Raf-1, but upstream of MEK. The Ras-MAPK signaling pathway controls cell growth, differentiation and survival. The pathway begins on the membrane surface and dispatches signals to the nucleus through a series of effectors proteins. Mutations in components of this signaling pathway underlie tumor initiation in mammalian cells [14,52]. This makes the Ras-MAPK pathway an appropriate target for anticancer therapy. Indeed, small molecular weight inhibitors designed to target various steps of this pathway are currently on the market and others are under evaluation in clinical trials. For example, sorafenib (Nexavar; Bayer HealthCare Pharmaceuticals), a Raf-kinase and VEGFR inhibitor that inhibits Ras signaling, is approved for the treatment of renal cell carcinoma [53]. Supported by these data, there is a continued high level of interest in targeting the Ras-MAPK pathway for the development of improved cancer therapies.

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