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Targeting the YAP-TEAD interaction interface for therapeutic intervention in glioblastoma

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Abstract

Introduction—Recent studies have suggested that dysregulated Hippo pathway signaling may contribute to glioblastoma proliferation and invasive characteristics. The downstream effector of the pathway, the Yes-associated protein (YAP) oncoprotein, has emerged as a promising target in glioblastoma multiforme (GBM).

Methods—Utilizing a high-throughput yeast two-hybrid based screen, a small molecule was identified which inhibits the association of the co-transcriptional activator YAP1 and the TEA domain family member 1 (TEAD1) transcription factor protein–protein interaction interface. This candidate inhibitor, NSC682769, a novel benzazepine compound, was evaluated for its ability to affect Hippo/YAP axis signaling and potential anti-glioblastoma properties.

Results—NSC682769 potently blocked association of YAP and TEAD in vitro and in GBM cells treated with submicromolar concentrations. Moreover, inhibitor-coupled bead pull down and surface plasmon resonance analyses demonstrate that NSC682769 binds to YAP. NSC682769 treatment of GBM lines and patient derived cells resulted in downregulation of YAP expression levels resulting in curtailed YAP-TEAD transcriptional activity. In GBM cell models, NSC682769

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Ethical approval Animal experiments were performed under an approved Institutional Animal Care and Use Committee protocol and conformed to guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

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inhibited proliferation, colony formation, migration, invasiveness and enhanced apoptosis. In tumor xenograft and genetically engineered mouse models, NSC682769 exhibited marked anti-tumor responses and resulted in increased overall survival and displayed significant blood-brain barrier penetration.

Conclusions—These results demonstrate that blockade of YAP-TEAD association is a viable therapeutic strategy for glioblastoma. On the basis of these favorable preclinical studies further clinical studies are warranted.

Keywords

YAP; TEAD; Hippo signaling; Small molecule inhibitor; Glioblastoma

Introduction

Glioblastoma is the most aggressive of CNS tumors with the median survival of 12–17 months, necessitating the development of novel therapeutics [1]. Aberrant regulation of the Hippo signaling pathway has been observed in several cancers including glioblastoma [2, 3]. Gene regulatory network and comprehensive expression analyses of brain tumor samples found that YAP and the transcriptional coactivator with PDZ-binding motif (TAZ) were major drivers of GBM transformation [4-6]. Additionally, clinical studies have demonstrated high expression levels of YAP in aggressive glioma subtypes (classical and mesenchymal) and elevated nuclear expression was associated with poor survival [5, 7].

The Hippo signaling pathway regulates tissue homeostasis and organ size [8]. Hippo (Mst1/2 in mammals) is activated by the NF2/Merlin-Kibra-Expanded tumor suppressor complex and phosphorylates the large tumor suppressor kinases (Lats1/2) leading to their activation [9]. The Lats1/2 kinases phosphorylate YAP resulting in its cytoplasmic retention and degradation [10]. Upstream growth control signals such as cell–cell, cell–matrix or extracellular soluble factors promote YAP and its paralog TAZ to concentrate within the nucleus where they co-activate TEAD transcription factors [11]. Activation of the TEADs (TEAD1-4) initiates expression of cellular communication network (CCN) matricellular protein family, such as connective tissue growth factor (CTGF) and Cyr61 [12, 13]. Expression of these growth factors promotes cell growth, migration, invasive potential and apoptosis avoidance [13-15].

Although significant evidence exists that YAP promotes tumor growth and metastasis through its interaction with its TEAD interaction domain, direct small molecule inhibitors of this protein-protein interaction (PPI) which are potent and specific are lacking. The identification of these types of molecules is anticipated to be challenging given the large interaction interface and apparent absence of a well-defined druggable binding pocket [16], however this obstacle may be overcome through biological screening of large, diverse chemical libraries. Previous strategies to disrupt the YAP-TEAD interaction, led to the identification of verteporfin (VP) which was demonstrated to block the YAP-dependent transcriptional activity of TEAD [17]. Several reports have described efforts to target upstream cascade components to induce YAP/TAZ phosphorylation such as activators of the Mst1/Lats1 kinases [18], or the Lats1/2 kinases [19, 20], or inhibitors specific for the

tyrosine kinase Yes-1 [21]. Statins were also described to reduce the nuclear accumulation of YAP/TAZ via effects on HMG-CoA [22]. Several peptide mimetics have been described which are able to compete with TEAD binding to YAP [23]. A cyclic peptide derived from YAP (amino acids 84–100) [24], a hybrid peptide (SUPER TDU) derived from VGLL4 protein and YAP (amino acids 74–99) [25] and a cysteine-rich peptide TB1G2 [26] were all demonstrated to disrupt the YAP-TEAD interaction. Additional small molecule inhibitors reported to curtail the YAP-TEAD interaction include a class of isothiazole-1,1-dioxides [27], flufenamic acids [28], a class of 3-(alkylthio)-triazoles [29], a fused tricyclic compound (CA3) [30], and four chemical families identified by virtual screening efforts [31]. Recently, small molecules that bind to the TEAD4 palmitate pocket and form a covalent bond with a conserved cysteine disrupting the YAP1-TEAD4 interaction have been described [16].

Here we report the discovery of small molecules which block the association of the YAP1 and TEAD1 interaction interface. One of these compounds, NSC682769 was characterized and evaluated for its anti-GBM effects. Biochemical studies demonstrate that NSC682769 binds to YAP, and shown to abrogate YAP-TEAD mediated transactivation and markedly inhibited GBM cell growth, motility and invasiveness. The effects of NSC682769 were also evaluated in GBM cell line xenografts and transgenic (Tg) glioma models.

Materials and methods

Details regarding cell cultures, reagents, in vitro and in vivo protocols and data analyses are described in Online Resource 1 Supplemental Materials and Methods.

Results

Identification of small molecule inhibitors of the YAP-TEAD interface interaction

To identify small molecule inhibitors of YAP-TEAD association, we engineered a yeast two-hybrid screen to identify compounds which blocked the YAP1-TEAD1 interface interaction [32, 33]. Yeast which were dependent on the interaction of these two protein domains for growth under selective conditions were used to screen the NCI/DTP small molecule library (Fig. 1a). Shown in Fig. 1b, identified compounds that blocked growth under these conditions and counterscreened these compounds for inhibition of growth of a strain dependent on the SV40 large T-antigen and p53 interaction. Compounds which inhibited both interactions were omitted and presumed to be generally toxic. Additionally, some compounds were predicted to be toxic to mammalian cells based on their structure and not pursued. We chose to study NSC682769 (Fig. 1c) as it exhibited minimal toxicity to normal human neurons (Online Resource 2 Suppl. Fig. S2). NSC682769 demonstrated marked inhibition of His-tagged YAP1 (residues 50–171) and GST-TEAD1 (residues 194–411) association in an in vitro GST-pull down assay (Fig. 1d) in a concentration-dependent manner. Moreover, NSC682769 blocked the association of YAP1 with TEAD1 in immunoprecipitates of YAP1 from LN229 GBM cells treated with the inhibitor (Fig. 1e). To examine the mechanism by which NSC682769 inhibits YAP, we determined the ability of full-length human YAP1 to bind to NSC682769 cross-linked affinity beads prepared using a photo-cross-linking procedure [34]. Recombinant YAP was incubated with control, IRES-

J007 (negative control), or NSC682769-coupled beads and binding analyzed by immunoblotting for YAP (Fig. 1f). As observed, native YAP associated strongly with NSC682769 while no significant binding was observed in control uncoupled or IRES-J007 coupled beads. Finally, we examined the binding of NSC682769 to immobilized YAP using SPR analyses. As shown in Fig. 1g, NSC682769 bound YAP in a concentration-dependent manner and reached equilibrium rapidly. The K_d was determined from steady-state binding associations and calculated at 738 nM supporting a direct interaction between NSC682769 and YAP. These results demonstrate that NSC682769 directly binds to YAP blocking its association with TEAD.

NSC682769 inhibits YAP expression, YAP-dependent transcriptional activity and results in cytoplasmic localization in GBM

To examine the effects of NSC682769 on YAP activity we treated GBM lines LN229, T98G and the patient-derived line GBM39 and examined YAP expression levels. As shown in Fig. 2a, following an 18 h exposure with increasing concentrations of NSC682769, YAP levels decreased while TEAD levels were unaffected. The inhibition of YAP expression by NSC682769 was also evaluated by ELISA in LN299 and GBM39 cells (Fig. 2b). The calculated IC_{50}'s for YAP were 11.8 nmol/L in LN229 cells and 5.1 nmol/L in the patient-derived GBM39 cells. We then examined whether NSC682769 would inhibit the association of YAP with particular TEAD family members (TEAD1-4). LN229 cells stably expressing an HA-tagged YAP were treated with NSC682769 for 18 h and lysates immunoprecipitated with α-HA antibodies and immunoblotted for TEADs 1–4 and several other transcription factors reported to associate with YAP [35-38]. As shown in Fig. 2c, NSC682769 treatment inhibited the binding of all four TEAD members to YAP, while having no appreciable effects on SMAD7, p73, FOS or TBX5 association. We monitored the expression of two YAP-target genes, *CTGF* and *Cyr61*. Both of these transcripts were downregulated following exposure to NSC682769 in LN229 and GBM39 cells (Fig. 2d). To assess the effects of NSC682769 on YAP transcriptional activity, LN229 or GBM39 cells were transfected with a TEAD binding site-based reporter (HOP-flash) and treated with increasing concentrations of the inhibitor. As shown in Fig. 2e, YAP-TEAD transcriptional activity was markedly reduced in response to NSC682769. To determine if NSC682769 altered the cellular localization of YAP we initially performed cell fractionation experiments. LN229 or GBM39 cell were treated with NSC682769 and cytoplasmic and nuclear extracts immunoblotted for YAP. As shown in Fig. 2f, exposure of either LN229 or GBM39 cells to NSC682769 (50 nM, 6 h) resulted in a reduction of nuclear YAP and a concomitant accumulation of cytoplasmic YAP consistent with its marked reduction in expression at longer timepoints following exposure to the inhibitor. These results were also supported in a series of immunofluorescence microscopy experiments in which either LN229 or GBM39 cells were treated with NSC682769 (50 nM, 6 h) and YAP localization determined (Fig. 2g). As shown, in LN229 and GBM39 control untreated cells the amount of nuclear staining was determined to be 67 % and 53 %, respectively. However, in cells treated with NSC682769 the relative amount of nuclear YAP was reduced to 26 % and 19 %, respectively. Together these data suggest that NSC682769 inhibits expression, activity and results in cytoplasmic redistribution of YAP in GBM cells.

NSC682769 inhibits GBM proliferation, migration and invasive characteristics

To examine the anti-GBM properties of NSC682769 we determined its effects on cell proliferation. As shown in Fig. 3a, NSC682769 significantly inhibited the growth of LN229 and patient-derived GBM39 cells. To determine whether NSC682769 inhibited anchorage-independent growth we performed soft agar colony formation assays. As shown in Fig. 3b, clonogenic growth of both LN229 and GBM39 cells was inhibited by NSC682769. To determine if NSC682769 would affect cell migration, we determined the capacity of treated cells to cross a vitronectin or fibronectin-coated Boyden chambers relative to chambers coated with control BSA. NSC682769 inhibited the number of cells capable of migrating towards vitronectin or fibronectin-coated surfaces relative to control BSA-coated chambers (Fig. 3c). The ability of NSC682769 to inhibit GBM cell invasiveness was examined in Matrigel invasion assays. As shown in Fig. 3d, the inhibitor reduced the number of cells able to traverse Matrigel-coated membranes. Additionally, apoptosis was enhanced in LN229 and GBM39 cells treated with NSC682769 (Fig. 3e). These data demonstrate the significant anti-GBM effects of NSC682769 in vitro.

Increased YAP expression directly correlates with NSC682769 sensitivity in GBM cells

The relative expression of YAP is increased in gliomas and promotes tumor proliferation via enhanced YAP-TEAD interactions and promotion of YAP-target gene transcription [5-7]. To examine whether differences in YAP expression may alter sensitivity to NSC682769 we determined the relative expression of nuclear versus cytoplasmic YAP in a panel of GBM lines (Fig. 4a). Expression of nuclear YAP varied greatly and was normalized to levels displayed in U87 cells. Cytoplasmic levels of YAP were more uniform, although the H4 line harbored elevated levels of both nuclear and cytoplasmic YAP relative to U87 cells. The expression of nuclear YAP between the GBM lines is shown in Fig. 4b. H4 and LN229 cells displayed the highest levels of nuclear YAP, while the T98G, DBTRG-05MG and LN18 lines expressed low levels and U87 and M059J lines expressed intermediate levels. Cell viability was then determined using ATP-release assays on these lines over a wide range of NSC682769 concentrations and IC_{50}s calculated (Fig. 4c). As shown in Fig. 4d, a significant inverse relationship was observed between the IC_{50} for NSC682769 and the relative nuclear YAP expression for these lines. These data demonstrated that lines with elevated nuclear YAP expression were the most sensitive to the compound while those with low levels were relatively resistant. T98G cells were stably transfected with either an shRNA plasmid targeting YAP1 or with a mammalian expression construct to overexpress YAP1. These cells were also examined for NSC682769 sensitivity and as shown in Fig. 4e, cells in which YAP1 had been knocked down were relatively resistant to NSC682769 compared to the parental T98G line, however cells in which YAP1 was overexpressed were significantly more sensitive.

NSC682769 inhibits GBM tumor growth in xenografted mice

To examine the effects of NSC682769 in vivo, we tested the ability of the compound to inhibit the growth of LN229 xenografted tumors implanted subcutaneously in SCID mice. Following implantation, when tumors reached ~ 200 mm3, mice were randomized into treatment groups receiving vehicle, 5 mg/kg/day and 20 mg/kg/day of NSC682769. As

shown in Fig. 5a, mice undergoing therapy with NSC682769 at either dosing schedule displayed a significant reduction in tumor growth rate relative to mice receiving vehicle (5 mg/kg/day; 66 % inhibition at end of dosing period and tumor growth delay 11 days; 20 mg/kg/day; 83 % inhibition at end of dosing period and tumor growth delay of 16 days). Overall survival of mice at either dosing regimen of NSC682769 was markedly extended relative to vehicle treated mice (Fig. 5b). We did not observe any overt short or long-term toxicity or weight loss associated with either dosing schedule in the mice. Moreover, neither blood cell counts nor serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) or creatinine levels were affected by NSC682769 (Online Resource 3 Suppl. Fig. S3). We observed a significant reduction in harvested tumor weights from both dosing regimens as compared to vehicle as shown in Fig. 5c. The relative expression level of YAP was reduced in harvested tumors from NSC682769 treated animals (5 mg/kg; 20 mg/kg) as compared to vehicle treated animals consistent with the observed effects of the inhibitor in vitro (Fig. 5d; see also Fig. 3). Expression of the YAP-dependent genes *CTGF* and *Cyr61* was reduced in inhibitor treated groups relative to vehicle (Fig. 5e). A reduction in Ki-67 staining of harvested tumors from animals treated with NSC682769 was evident as compared to vehicle treated mice (Fig. 5f) and enhancement of apoptotic death was observed via TUNEL staining at both dosing regimens, supporting the increased rate of apoptosis observed in vitro (Fig. 5g, see also Fig. 3e).

NSC682769 increases survival of a glioma GEMM and penetrates the blood-brain barrier

We evaluated NSC682769 in double transgenic *GFAP-EGFRvIII; GFAP-Cre/Rictor*$^{loxP/loxP}$ mice which overexpress both the mutant constitutively active EGFRVIII allele and the mTORC2 scaffolding component Rictor. These mice develope high-grade bilateral, multifocal, infiltrating mixed astrocytic-oligodendroglial tumors displaying elevated mTORC2 signaling with nearly complete penetrance [39]. In a cell line derived from oligodendroglial tumors from these mice (R0E3 cells) YAP-dependent gene expression was elevated (Online Resource 4 Suppl Fig. S4). To assess therapeutic activity we measured the effect of twice-weekly 5 or 20 mg/kg NSC682769 treatments on survival. As shown in figure S5A (Online Resource 5 Suppl Fig. S5), approximately 50 % of *GFAP-EGFRvIII* × *GFAP-Cre*$^{+}$*/Rictor* mice develope gliomas by 5–7 weeks and all of the mice succumbing by 16 weeks. Importantly, NSC682769 treated *GFAP-EGFRvIII* × *GFAP-Cre*$^{+}$*/Rictor* mice had a marked increase in overall survival with more then 75 % of mice surviving at 20 weeks at 20 mg/kg and 60 % of mice surviving to 20 weeks receiving the lower 5 mg/kg regimen.

While GBMs may partially disrupt the BBB by inducing large gaps between endothelial cells, the extent of disruption among individual patients and/or among various regions within a single tumor are highly variable. Thus, we determined whether NSC682769 would cross the BBB by developing an HPLC method to quantify the inhibitor in brain tissue and serum. In C57/BL6 mice, following a single IV administration of NSC682769, the compound accumulated to significant levels with a peak value of 6.22 μg/g at 5 min post-administration (Fig. S5B). C_b/C_p ratios for NSC682769 are consistent with BBB penetrance.

Discussion

The Hippo signaling pathway has received considerable attention recently and has been implicated as an attractive therapeutic target in GBM [5, 38, 40, 41]. Evidence suggests that deregulation of Hippo signaling and activation of its downstream effector YAP leads to increased GBM cell growth and motility [5, 41]. In the current study we identified a potent small molecule inhibitor and demonstrated that it has marked inhibitory effects on YAP/TEAD transcriptional activity and anti-GBM properties on cell lines and patient-derived cells. NSC682769 appears to directly bind to YAP and inhibits its association with all four TEAD family members. Moreover, sensitivity to NSC682769 directly correlated with an elevated degree of nuclear YAP expression. NSC682769 also enhanced the survival of a transgenic glioma mouse model in a dose-dependent manner and determinations of mean brain and blood plasma concentrations following single IV administration of the inhibitor suggested clear blood-brain barrier penetrance.

Our data are consistent with a working model in which NSC682769 attenuates YAP expression by binding to YAP and resulting in targeted degradation of the protein. This is indirectly supported by both our cell fractionation and immunolocalization studies. In LN229 and GBM39 cells, treatment with NSC682769 resulted in a marked cytoplasmic redistribution of nuclear YAP (see Fig. 2f, g) where normally, the S127 phosphorylated protein binds to 14-3-3 proteins resulting in their cytoplasmic retention and suppressed target gene transcription. Further serine phosphorylation of YAP by casein kinase 1, leads to subsequent proteasomal degradation [10]. It is tempting to speculate that NSC682769-bound YAP adopts a conformation enhancing 14-3-3 binding, leading to proteasomal-mediated turnover.

It has been suggested that the development of orthosteric small molecule inhibitors of the YAP-TEAD protein-protein interface may be difficult owing to the interaction occurring over a large and mostly featureless interaction interface (~ 1300 $Å^2$) with a K_d in the nanomolar range [16]. While this may be the case, it is possible that small structural pockets may exist which are competent to bind small probe compounds. Our NSC682769 affinity bead pull-down and SPR analyses demonstrate that the compound binds to YAP (see Fig. 1f, g). In this regard, verteporfin, identified as a prototypic YAP-TEAD interaction inhibitor [17], has not been demonstrated to bind either YAP or TEAD and its inhibitory effects on YAP signaling may be due to non-specific effects [42]. While verteporfin did inhibit YAP-TEAD association in our in vitro experiments, the blockade required markedly higher levels of verteporfin to interfere with the interaction as compared to NSC682769 exposure (see Fig. 1e, *and legend description*). Future binding site mapping, in silico docking and mutagenesis experiments will shed light on the detailed mechanism of action of NSC682769.

In conclusion, our studies demonstrate that NSC682769 represents a newly identified inhibitor of the YAP-TEAD protein-protein interaction targeting GBMs with elevated YAP expression. NSC682769 appears to have significant inhibitory effects on YAP-TEAD dependent transcription and markedly inhibits GBM cell growth. Experiments investigating

this compounds structure-function relationships in order to enhance inhibitory activity or optimize ADMET properties are merited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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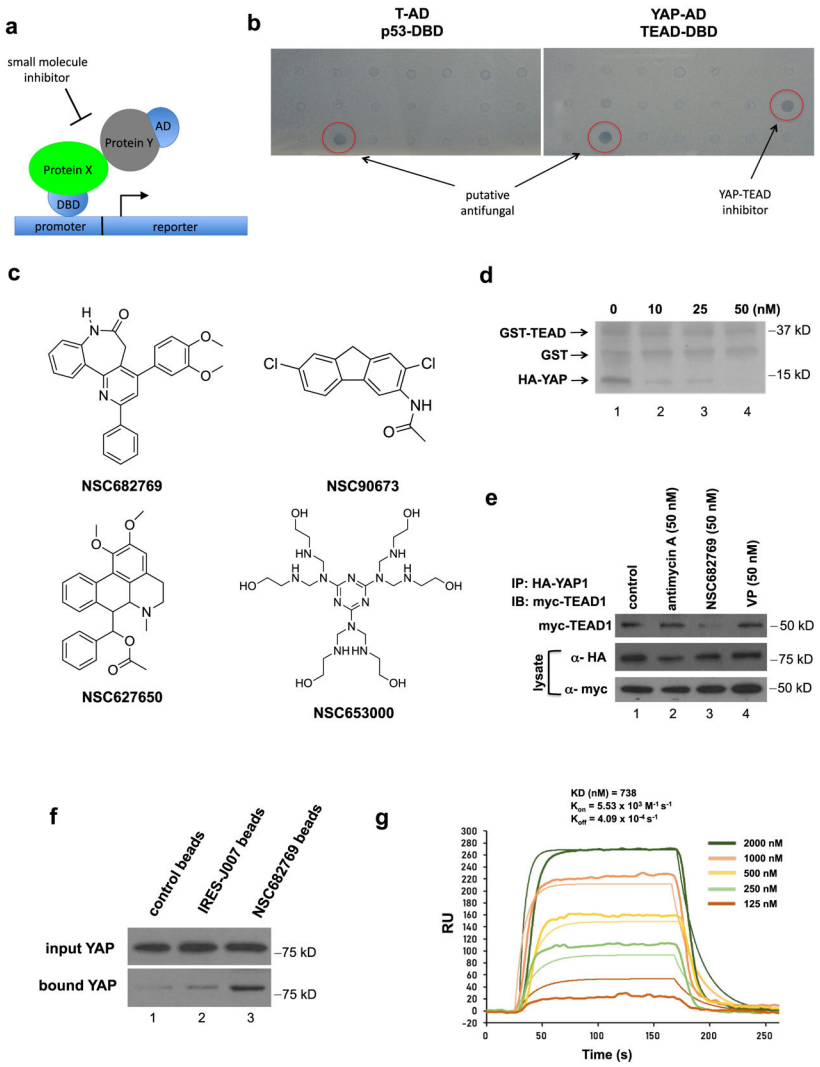

Fig. 1.

Identification of a YAP1-TEAD1 binding interface inhibitor. **a** Schematic diagram of the yeast two-hybrid configuration used for screening. The Gal4 DBD was fused to residues 50–171 of human YAP1 and the Gal4 AD was fused to residues 194–411 of human TEAD1. These fusions were expressed in yeast containing reporters harboring *Gal4* upstream activating sequences (UAS). **b** High-throughput screening of compounds which inhibit YAP1-TEAD1 association. Yeast expressing either p53-DBD and SV40 large T antigen-AD fusions or YAP1-DBD and TEAD1-AD fusions were plated on selective media. Compounds were pinned onto the plate surfaces by robotic transfer and subsequently examined for halo formation (indicated by red circles). Compounds inhibiting YAP1-TEAD1 mediated growth on selective media while having no affect on p53-T antigen mediated yeast growth were considered specific. Compounds which blocked the growth of both strains were considered nonspecific and were classified as having general antifungal properties. **c** Compound structures of candidate YAP-TEAD inhibitors. **d** NSC682769 blocks in vitro YAP-TEAD binding in a GST pull-down assay. His-tagged YAP (a.a. 50–171) was used in a GST pull-down assay using GST-TEAD (a.a. 194–411) immobilized on glutathione resin. Pull-down

products were analyzed by SDS-PAGE and silver staining. **e** LN229 cells expressing HA-YAP and myc-TEAD were incubated with the indicated concentrations of inhibitors for 24 h and the presence of myc-TEAD in the HA-YAP immunoprecipitates was probed. Cells treated with antimycin A is shown as a negative control. Note verteporfin (VP) at equimolar concentrations was ineffective at inhibiting the interaction and required cell treatment at 4 μM for observable blockade (data not shown). **f** Purified recombinant HA-tagged full-length human YAP1 was added to uncross-linked control, IRES-J007 (hnRNP A1 inhibitor) and NSC682769 coupled beads and the amount of bound YAP was assessed by immunoblot analyses. **g** Surface plasmon resonance analyses of immobilized full-length YAP binding to the indicated concentrations of NSC682769 analyte. Raw binding sensorgrams obtained at each concentration were fitted to a 1:1 binding model and K_{on}, K_{off} and KD were calculated by simultaneous non-linear regression analyses

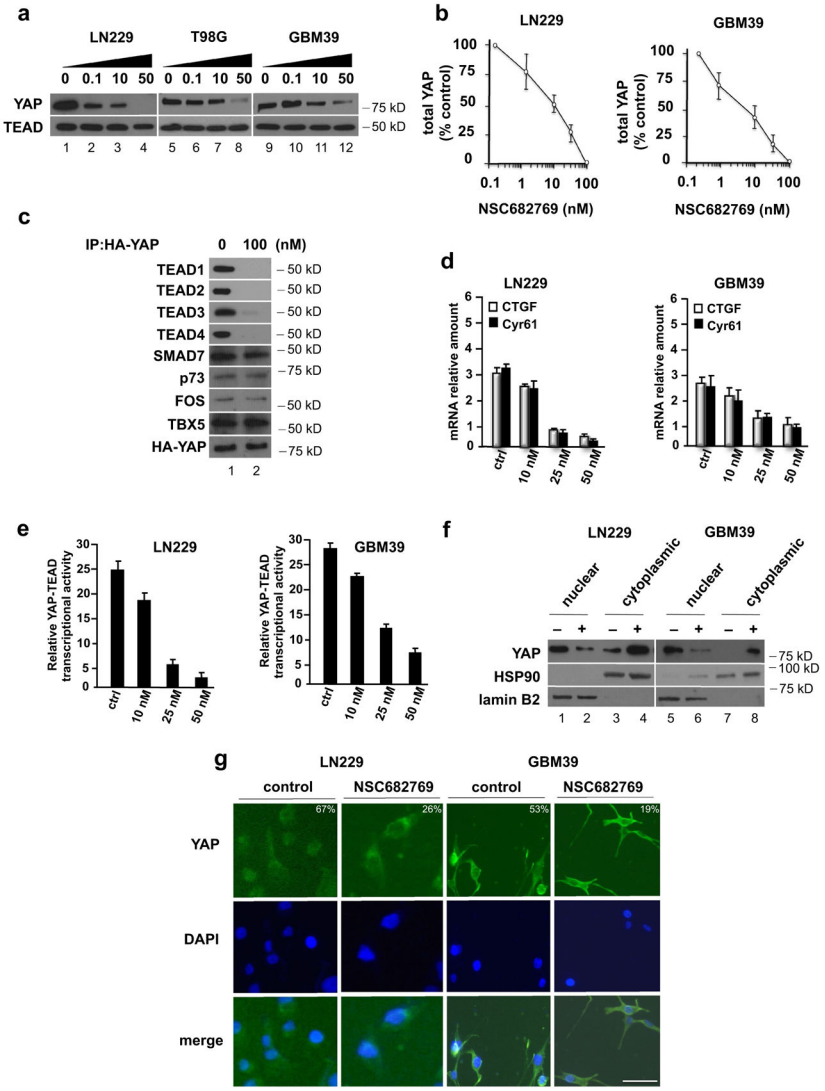

Fig. 2.

NSC682769 selectively inhibits YAP-TEAD signaling in GBM. **a** Effects of NSC682769 following 18 h incubation at the indicated concentrations in LN229, T98G and the patient-derived GBM line GBM39. **b** Inhibition of YAP expression in LN229 and GBM39 cells. Levels of total YAP were determined via ELISA. NSC682769 inhibited total YAP with IC_{50}'s of 11.8 and 5.1 nM, in LN229 and GBM39 cells, respectively. **c** LN229 cells expressing HA-tagged YAP1 were treated with NSC682769 for 18 h as indicated and α-HA antibody used to immunoprecipitate HA-YAP1. Immunoprecipitates were immunoblotted for the indicated proteins. **d** Inhibition of YAP-target gene (*CTGF* & *Cyr61*) expression by NSC682769 in LN229 and GBM39 cells. Cells were treated with the indicated concentrations of inhibitor for 18 h and mRNA extracted for real time RT-PCR analyses. Measurements were performed in quadruplicate and means and + S.D. are shown. **e** LN229 or GBM39 expressing a multimerized TEAD binding site luciferase reporter (HOP-flash) were treated with the indicated concentrations of NSC682769 for 18 h and YAP-TEAD transcriptional activity determined via luciferase assays. f Effects of NSC682769 on YAP

cytoplasmic/nuclear accumulation in LN229 and GBM39 cells. Cells were treated with (+) or without (−) NSC682769 (100 nM, 18 h) and harvested and separated into cytoplasmic and nuclear cellular fractions. Cytoplasmic versus nuclear material was subsequently immunoblotted for the indicated proteins. **g** Indirect immunofluorescence analyses of YAP localization following exposure of LN229 and GBM39 cells to NSC682769 (50 nM, 6 h). Values in top right corner of panels correspond to the percentage of nuclear localized YAP. Cells were grown on coverslips and treated with NSC682769. Cells were permeabilized and stained using primary antibody to YAP1 and FITC-conjugated secondary. Nuclei were stained with DAPI. Scale bar, 20 μm

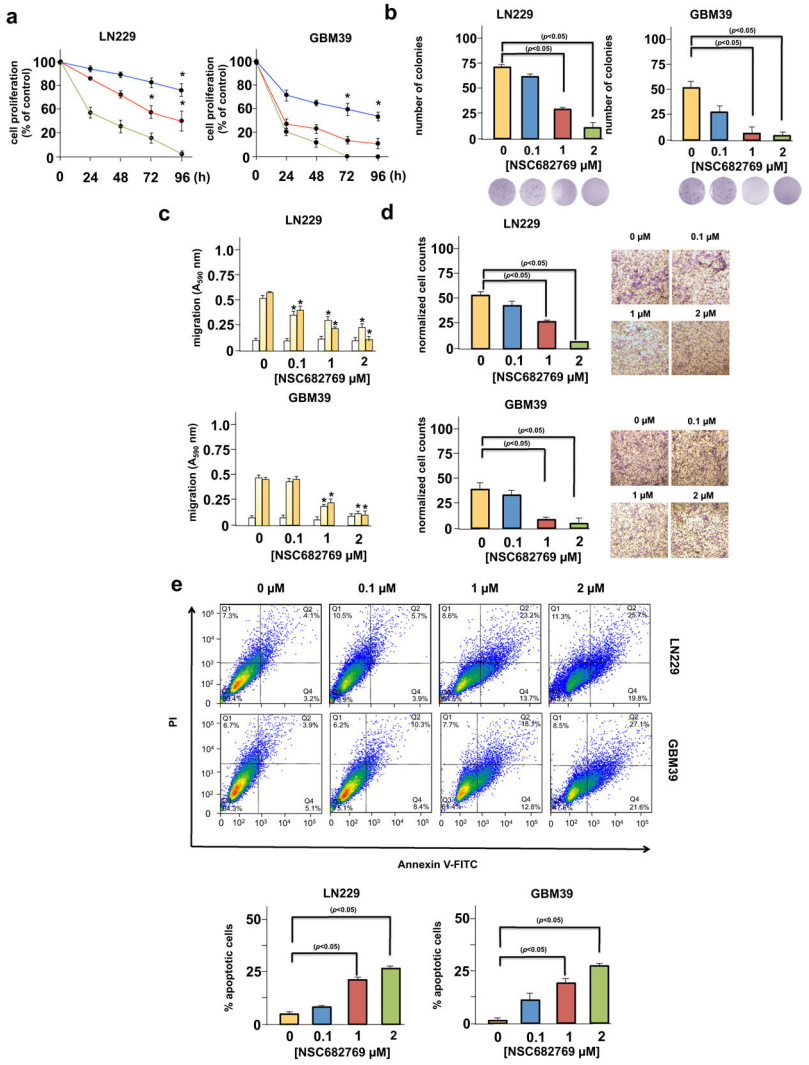

Fig. 3.

NSC682769 inhibits proliferation, anchorage-independent growth, motility, invasive potential and induced apoptosis in GBM. **a** Inhibition of LN229 and patient-derived GBM line GBM39 proliferation following culture with NSC682769 (blue, 0.1 μM; red, 1 μM; green, 2 μM) for the indicated time points (*$P < 0.05$). **b** Inhibition of anchorage-independent growth by NSC682769. Cells were layered in soft agar to evaluate anchorage-independent growth in the presence of the indicated concentrations of inhibitor and colonies counted following 14 days of growth. Representative crystal violet stained images are shown below. Data represent mean + S.D. of three independent experiments. **c** Migration of LN229 and GBM39 cells in the presence of NSC682769 at the concentrations shown. Cells were placed in Boyden chambers and allowed to migrate towards BSA (white bars), vitronectin (light yellow bars), or fibronectin (dark yellow bars) (*$P < 0.05$; n = 3). **d** Invasive potential of LN229 or GBM39 cells in the presence of the indicated concentrations of NSC682769 migrating through matrigel. Representative crystal violet stained images are shown (200×). Data represent mean + S.D. of three independent experiments. **e** Percent apoptotic cells in LN229 and GBM39 cells treated with the indicated concentrations of NSC682769 at 48 h as

determined via annexin V-FITC/PI staining and flow cytometry. Graphical data shown below represent mean + S.D. (late apoptosis; annexin V-positive, PI-positive) of three independent experiments

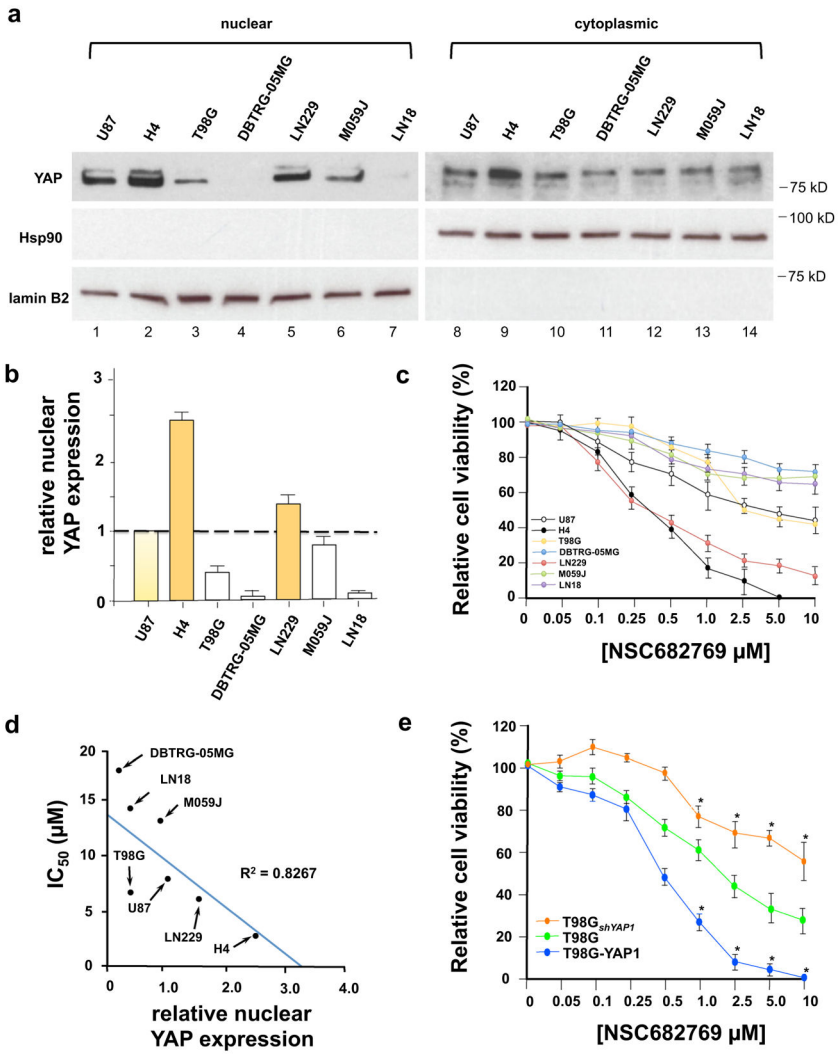

Fig. 4.

Elevated nuclear YAP levels correlate with NSC682769 sensitivity in GBM. **a** Nuclear and cytoplasmic accumulation of YAP in the indicated GBM lines. Nuclear and cytoplasmic fractions were immunoblotted for YAP, HSP90 and lamin B2. **b** Quantification of nuclear YAP expression levels from **a** as determined by densitometric analyses. Relative nuclear YAP1 expression in U87 cells was arbitrarily assigned a value of 1. Nuclear YAP expression in all cell lines is shown as mean + S.D., n = 3. **c** Relative cell proliferation was determined via Cell Titer-Glo® luminescent assays on the indicated GBM lines with increasing concentrations of NSC682769 at 72 h. **d** Correlation between NSC682769 IC_{50} and relative nuclear YAP expression as determined for all GBM cell lines treated with NSC682769 for 72 h and shown as means of 3–5 individual experiments. Relative nuclear YAP expression was obtained from **b** above. **e** Differential sensitivities of parental T98G, shRNA YAP expressing T98G and hYAP1 overexpressing T98G cells exposed to NSC682769 at the indicated concentrations at 72 h. Data represent mean ± S.D. of three independent experiments (*$P < 0.05$)

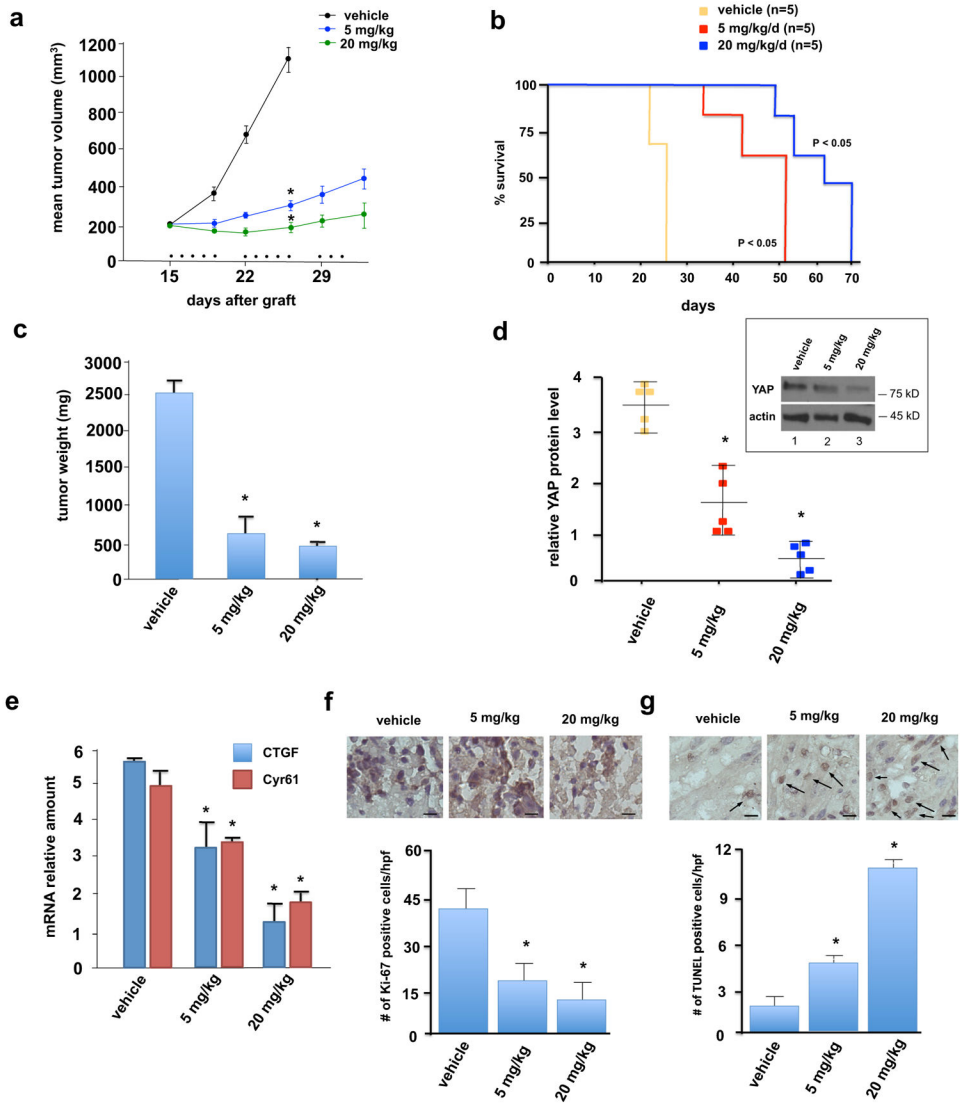

Fig. 5.

NSC682769 inhibits GBM tumor growth in mice. **a** Effects of NSC682769 on tumor growth in SCID mice implanted with subcutaneous LN229 xenografts and treated with the indicated schedules of vehicle, NSC682769 (5 mg/kg/day) and NSC682769 (20 mg/kg/day). ·, treatment day; **P* < 0.05, significantly different from vehicle (n = 5 per group). **b** Overall survival of mice harboring subcutaneous LN229 tumors receiving the indicated treatment schedules of NSC682769. **c** Tumor weights of harvested tumors from xenografted mice treated with the indicated treatment schedules of NSC682769. **P* < 0.05, significantly different from vehicle. **d** YAP protein levels in tumors from harvested tumors. Protein levels were quantified by Western analyses (*inset*; representative immunoblots of YAP and actin levels from tumors from the indicated tumor treatment groups) and normalized to actin levels of harvested tumors from mice with the corresponding treatments as indicated and described in Materials and Methods. Values are means ± S.D., **P* < 0.05, significantly different from vehicle, NSC682769 (5 mg/kg/day) and NSC682769 (20 mg/kg/day). **e** Inhibition of YAP-dependent gene transcription in harvested xenografts treated with the

indicated regimens. *CTGF* and *Cyr61* mRNA levels were determined via real-time RT-PCR measurements. **P* < 0.05 significantly different from vehicle, NSC682769 (5 mg/kg/day) and NSC682769 (20 mg/kg/day). **f** Ki-67 positive cells were identified via immunohistochemical staining of sections prepared from harvested tumors at day 12 following initiation of treatment regimens. **P* < 0.05 significantly different from vehicle. Scale bar, 20 μm. **g** Apoptotic cells were identified by TUNEL assays of sections prepared from harvested tumors at day 12 following initiation of treatment regimens. Data are expressed as the number of positive apoptotic bodies (brown, indicated by arrows) divided by high power field (hpf; 10–12 hpf/tumor). Values are means + S.D., **P* < 0.05. Scale bar, 20 μm