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Solution phase parallel synthesis and evaluation of MAPK inhibitory activities of close structural analogues of a Ras pathway modulator

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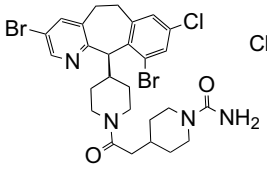
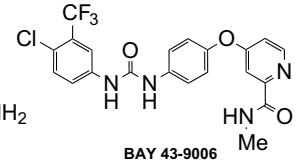
Abstract—A solution phase parallel synthesis approach was undertaken to rapidly explore the structure–activity relationship of an inhibitor of the Ras/Raf protein interaction identified from a small molecule compound library. Evaluation of the MAPK pathway signaling inhibitory activity of the synthesized analogues as well as their antiproliferative activity and ability to inhibit soft agar growth were performed.

The mitogen activated protein kinase (MAPK) pathway controls many critical cellular functions such as cell growth, cell differentiation and apoptosis.[1] Unregulated activation of Ras (derived from *rat sarcoma*), a G-protein involved proximally in this signal transduction pathway, is thought to be a significant contributor to cancer development, particularly colon and pancreatic cancers.[2] As a consequence, for over a decade, proteins constituting this cell signaling pathway have been intensely investigated as potential biological targets for the discovery of novel, mechanism based anticancer agents.[3,4]

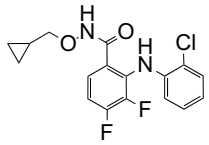
An initial step in Ras activation is Ras farnesylation, catalyzed by the enzyme Ras farnesyltransferase and postulated to be essential for anchoring inactive cytosolic Ras to the cell membrane.[5] Much effort has been expended towards developing novel antitumor compounds via inhibition of this enzyme. Several farnesyltransferase inhibitors emanating from such efforts, among them SCH66336 (Lonafarnib™) and R115777 (Tipifarnib™) are currently reported to be undergoing advanced clinical trials.[4,6]



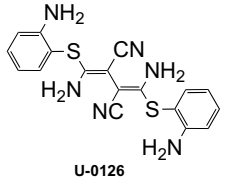
SCH66336



BAY 43-9006



PD-184352



U-0126

Other groups working on developing anticancer agents targeting the MAPK pathway have focused their research efforts at inhibiting one of the intrinsically key mechanistic processes in cell signal transduction, namely protein phosphorylation.[6] Apart from the clinically well established EGFR kinase inhibitors such as Iressa™ and Tarceva™, one of the more functionally characterized,

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newer representatives of this class of small molecule modulators of the MAPK pathway is BAY 43-9006 (Sorafenib™), a Raf kinase inhibitor currently in Phase III clinical trials in patients with advanced renal cell carcinoma.[7] Others of note include the MEK inhibitors PD-184352, U-0126, and Ro 09-2210.[4,6] Also, an antisense oligonucleotide inhibitor of cRaf kinase gene expression, ISIS-5132, has demonstrated in vivo antitumor activity in animal models.[4,6]

Conceptually, within the framework of the MAPK protein signaling cascade, a small molecule inhibitor of the Ras/Raf protein interaction could also be envisioned to be a novel and effective mechanism based cancer chemotherapeutic agent.[8] In this regard, the interaction between Ras and Raf has been shown to be inhibited in vitro by heptapeptides derived from either Ras or Raf[9] and by Sulindac sulfide.[10] Since nonpeptidic small molecule inhibitors can have distinct pharmacokinetic advantages over peptidic inhibitors, we had previously undertaken and subsequently reported on the identification of such compounds from the screening of a small molecule compound library.[11] Our efforts toward investigating the structure–activity relationship (SAR) as well as the attempted concomitant optimization of the medicinal chemical properties of one such screening hit is the topic of this communication.

Utilizing a yeast two-hybrid primary screen, **1** was identified as an effective and specific inhibitor of the interaction of Ras and Raf.[11] Subsequently, in a SRE-luciferase reporter assay in mammalian CHO cells measuring a reduction in serum induced transcriptional activation of serum response element, **1** was characterized to exhibit weak but reproducible activity ($IC_{50} = 17.9\,\mu M$).[11] A meaningful assessment of the ability of **1** to inhibit the Ras/Raf protein interaction complex in vitro was prohibited in part by the difficulty encountered in purifying full length Raf in its natively folded form as well as the complexity associated with mimicking a functional multi-protein complex in a cell-free system.

Physicochemically, **1** may be categorized as more 'drug-like' than 'lead-like'[12] given the fact that it exhibits a high MW (487) and log P[13] (5.610); these physicochemical parameters also lie at the higher end of an optimal range prescribed for orally active drugs based on the Lipinski 'rule of 5'.[14] Considering in addition the weak cellular activity of **1**, one critical question we needed to immediately address was whether **1** was indeed a 'drugable' candidate, exhibiting a clear SAR. Structurally **1** represents a synthetically readily accessible chemotype, amenable to multiple structural modifications via solution phase parallel synthesis such that analogues can be synthesized and evaluated rapidly in high purity. With these considerations in mind, and further, taking into account the demonstrated reversion of Ras-transformed phenotypes exhibited by **1**,[11] we decided to embark on a course of preliminary structure–activity studies by conservatively modifying **1** through analogue derivatives. We rationalized that if a clear SAR and a modest improvement in potency and/or aqueous solubility could be demonstrated through the synthesis of an initial set of analogues, this could possibly provide an impetus for future focused efforts directed toward rendering **1** more potent ($<5\,\mu M$ cellular activity) as well as more water soluble ($\log P < 4$).

Some of the initial structural modifications of **1** that we envisioned are illustrated in Figure 1. For the sake of description, **1** has been structurally categorized into three components attached to the central amide functionality: an aryl amide moiety, a benzylamine moiety, and a pyridylethylamine moiety. Depicted in Scheme 1 is the flow chart for the solution phase parallel synthesis of **1** analogues. In short, analogues of **1** were readily synthesized by a two-step protocol involving an initial sequential reductive amination reaction followed by acylation of the resultant secondary amines. Parallel purification of the reaction mixtures were typically accomplished by the use of scavenger resins,[15] with or without subsequent ion-exchange chromatography,[16] to afford the desired products in >80% purity, as determined by LC–MS.[17] Parallel solution phase syntheses were regularly run on a 100–300 mg scale of starting reactants so as to also enable archiving of compounds. For larger scale (gram amount) preparations of the secondary amine, $NaBH_4$ was employed as the imine reducing agent and the reactions were worked up in the traditional manner. Minor unwanted formation of the corresponding tertiary amine side product in the reductive amination reaction was successfully suppressed by employing a slight excess of the primary amine reagent with respect to the aldehyde component. In reactions involving acylation of the secondary amine with an isocyanate to provide an urea derivative, polymer supported morpholine was excluded. Where necessary, free carboxylic acid or free amino functionalized reagents were utilized as their methyl ester or *N*-*tert*-butyloxycarbonyl (Boc) protected forms, respectively. Regeneration of the free acid or free amine from the corresponding protected form was accomplished utilizing standard protocols.[18]

Analogues of **1** synthesized in this study were initially screened at 20 μM final concentration in an Elk1-luciferase reporter assay in HeLa cells[19] and compounds

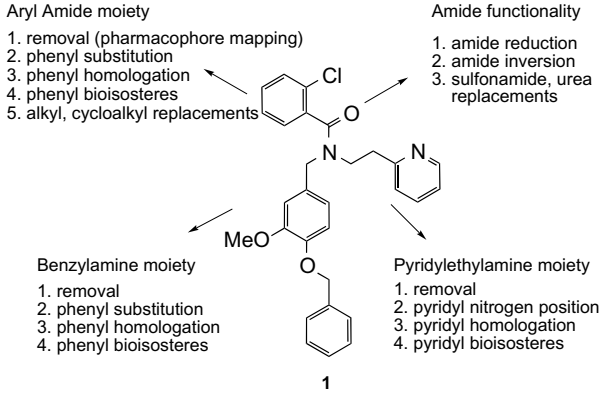
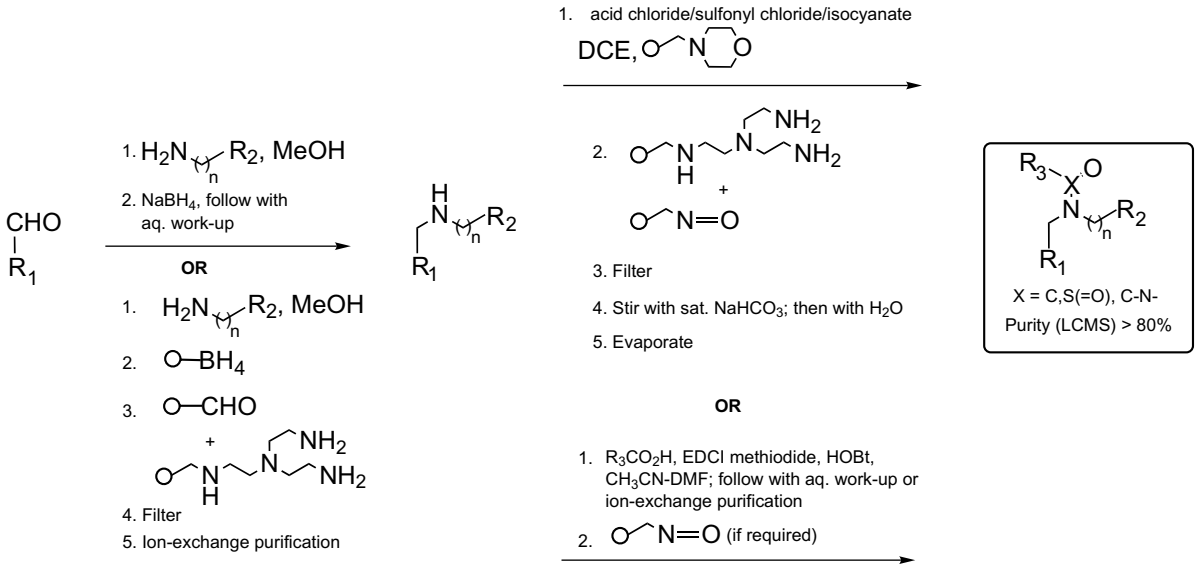


Figure 1. Conservative structural modifications of **1**.



Scheme 1. Solution phase parallel synthesis of **1** analogues.

exhibiting $\geqslant$50% inhibition were further profiled in this assay to obtain an IC_{50} measurement. Analogues exhibiting interesting levels of inhibition ($IC_{50} < 20\,\mu M$) in the Elk1-luciferase assay were subsequently evaluated for their antiproliferative activity (WST-1 assay)[20] as well as their ability to inhibit anchorage independent colony formation (soft agar growth assay) in HCT-116 cells.[21] Data for selected analogues shown in Figure 2 is listed in Table 1.

Our observations regarding the SAR garnered from the synthesized series of **1** analogues are outlined below. Unless otherwise noted, the compound activity (or lack thereof) refers to the inhibitory activity of the **1** analogue in the Elk1-luciferase assay.

Of note, truncated analogues of **1** lacking either one of the three structural components attached to the central amide functionality (vide supra and Fig. 1) were observed to exhibit significantly reduced activity (data not shown), suggesting that **1** was the minimal pharmacophore. Significantly, this rendered the possibility of simultaneously improving potency and reducing lipophilicity of **1** more difficult. Reduction of the amide functionality of **1** to afford the corresponding tertiary amine resulted in a loss in activity. Amide inversion of **1** as in analogues **2** or **3** was well tolerated.

Substitution of a sulfonamide or urea functionality for the amide group was found to be detrimental to the compound activity.

On the aryl amide moiety of **1**, the aryl halogen substituent was observed to be necessary for activity. Substitution of the phenyl ring at all three ring positions with groups exhibiting a range of steric and electronic properties[22] was explored. In general, lipophilic substituents were better tolerated than hydrophilic substituents on the ring; however, where tolerated, activities of the corresponding analogues were mostly clustered. Replacement of the phenyl ring with alkyl, cycloalkyl, or heterocyclic groups (e.g., thiophene or pyridyl) did not augment activity, rather, this even proved to be detrimental in some instances. Utilization of phenyl substituted amino acid derivatives (e.g., D- or L-Phe, **4**) as the amide component, as a means to improve water solubility, also proved detrimental. Significantly, homologation of the aryl amide moiety improved activity with a four carbon spacer, **5**, demonstrating enhanced activity. Compounds **6** and **7** bearing a heteroatom such as O or N, respectively, in an extended aryl amide linker exhibited weaker activity. Also, introduction of a conformational restriction element such as an olefin functionality or a cycloalkyl group (cf. **8**) into the linker to favor an extended linker conformation as well as chain branching of the linker as in **9** were without added beneficial effect on compound activity relative to **5**. The inhibitory activities exhibited by **1** and **5** in the soft agar assay is illustrated in Figure 3.

Compounds lacking the benzyloxy substituent on the benzylamine moiety (cf. **10**) typically demonstrated loss of activity. Removal of the methoxy substituent in some cases proved to be nondetrimental to activity. We posit that this site on the molecule could serve as a point of attachment of solubilizing groups (cf. **11** and **12**) and further investigation of the scope and limitations of such substitutions is clearly warranted. Positional interchange of the methoxy and benzyloxy substituents on the phenyl ring as in **13** lead to decreased activity. Replacement of the benzylamine phenyl ring with a piperidine moiety such as in **14** abolished activity.

With regards to the pyridylethylamine moiety of **1**, positional changes of the nitrogen atom as well as its elimination lead to significant reduction in activity. Attempts at reducing the overall lipophilicity of **5** through differential oxidation of the pyridine ring to a pyridone **15** or a pyridine *N*-oxide lead to a reduction in activity of

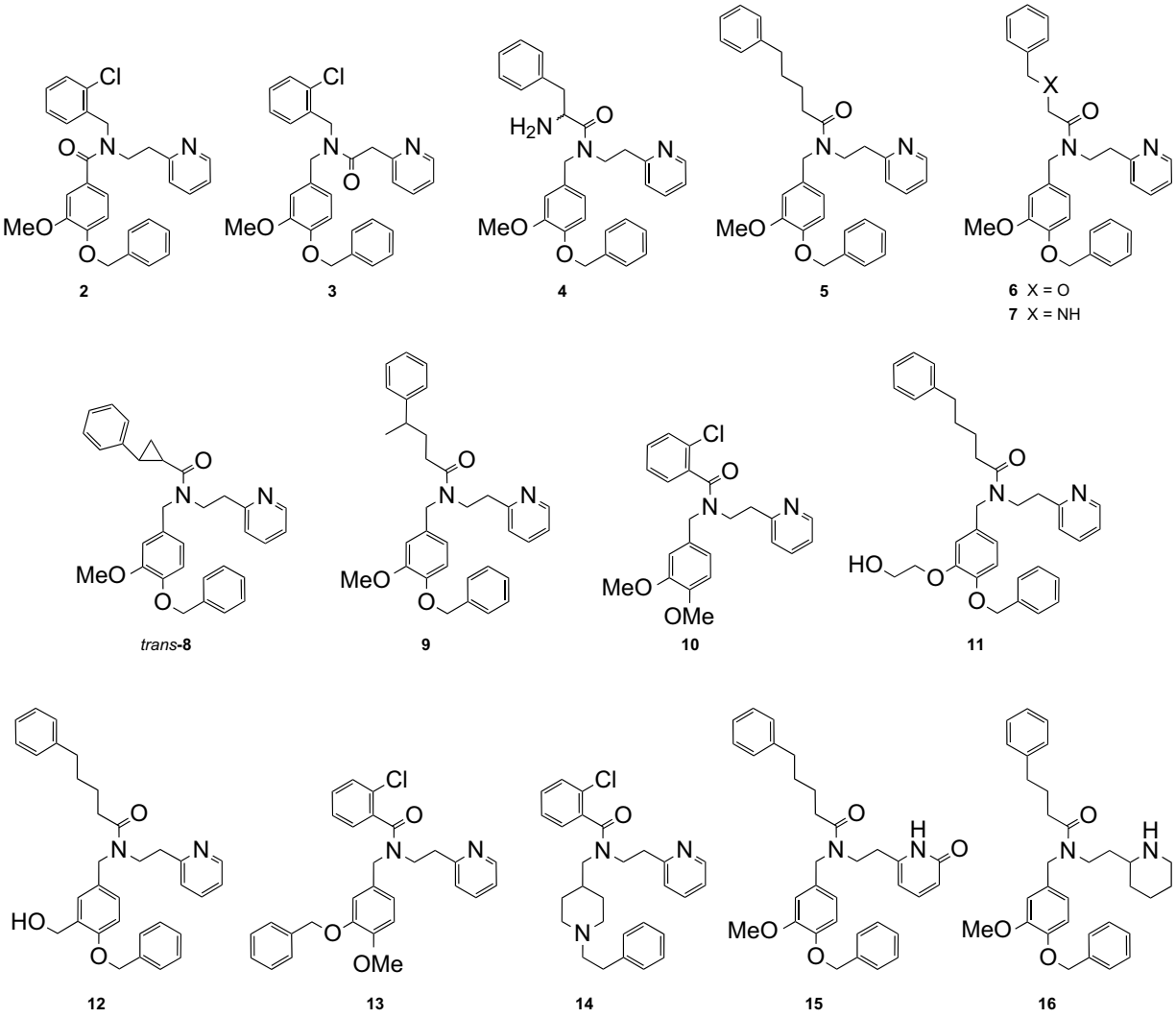


Figure 2. Selected analogues of **1**.

Table 1. Representative data for **1** and selected analogues

Compound	ELK1 IC_{50} (μM)	Proliferation (HCT-116) GI_{50} (μM)	Soft agar (HCT-116) GI_{50} (μM)
1	20.6 ± 3.8	24.7 ± 3.4	22.1 ± 3.2
2	16.2 ± 3.1	29.0 ± 4	18.8 ± 1.5
3	25.3 ± 3.4	ND[a]	ND
4	14% Inhibition @ 20 μM	ND	ND
5	9.0 ± 0.6	16.2 ± 3.7	20.6 ± 2.8
6	43% Inhibition @ 20 μM	ND	ND
7	6% Inhibition @ 20 μM	ND	ND
8	10.5 ± 0.6	23.9 ± 5.7	25.9
9	11.2 ± 4.3	13.2 ± 4.5	23.5
10	24% Inhibition @ 20 μM	ND	ND
11	14.2 ± 4.6	18.6 ± 1.7	14.4 ± 1.3
12	19.4 ± 3.1	31.6 ± 5.6	ND
13	32% Inhibition @ 20 μM	ND	ND
14	7% Inhibition @ 60 μM	23% Inhibition @ 60 μM	ND
15	12.3 ± 1.2	22.6 ± 5.2	32.3 ± 2.2
16	0% Inhibition @ 15 μM 100% Inhibition @ 30 μM	5.4 ± 0.1	3.5 ± 1.1
BAY 43-9006[b]	3.3 ± 0.4	5.4 ± 0.4	6.6
U-O126[b]	0.29 ± 0.03	35% inhibition @ 15 μM	19.4

[a] ND, not determined.
[b] Commercially available from Calbiochem; http://www.merckbiosciences.com/html/CBC/home.html.

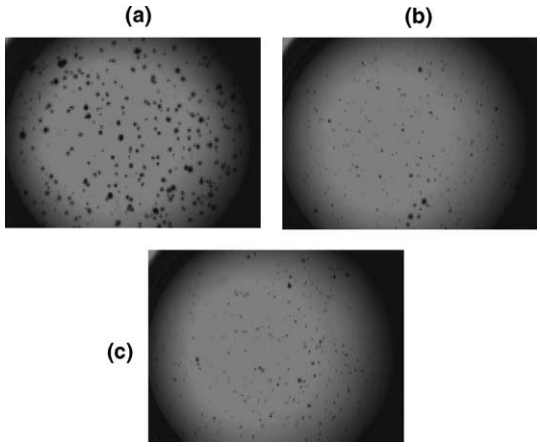


Figure 3. Inhibition of anchorage-independent growth in HCT-116 cells by (a) DMSO control (b) **1** (20 μM) and (c) **5** (20 μM).

the resulting compounds. Alternatively, replacement of pyridine with piperidine as in **16** lead to an undesirable, nondose dependent inhibition of Elk1-luciferase activity; however, interestingly, **16** exhibited significant potency improvement in the proliferation and soft agar assays, presumably through interfering with other cellular signaling mechanisms or through nonspecific effects.

In summary, roughly 300 conservatively modified analogues of the Ras/Raf protein interaction inhibitor **1** were rapidly synthesized utilizing solution phase parallel synthesis techniques with the initial objective of elucidating a meaningful SAR within that analogue series. From the present set of **1** analogues, we surmise that at the aryl amide and the benzylamine moieties, hydrophobic elements are critical determinants of the effectiveness of these compounds in inhibiting the MAPK signaling pathway (Fig. 4). The necessity of the amide functionality for compound activity may reflect the importance of a hydrogen bonding interaction at this site and/or the requirement for an appropriate conformational restraint in place. The replacement of the methoxy group on the benzylamine moiety with aqueous solubility enhancing groups might serve to decrease the overall lipophilicity of these molecules while maintaining or improving inhibitory potency. It appears that a hydrogen bonding functionality on the pyridylethylamine moiety is critical to the inhibitory activity of **1** and its analogues in the MAPK pathway. Overall, a modest improvement in potency has resulted within the synthesized analogue series, a preliminary SAR has been elucidated, and further, possible ways to enhance the aqueous solubility of these compounds have been delineated. Also, taking into account the abundant representation of phenyl rings in **1** in addition to its intrinsic conformational flexibility, the possibility of a hydrophobically collapsed[23] conformation being critical to the inhibitory activity of **1** in the MAPK pathway cannot be ruled out. Evaluation of this hypothesis is currently underway through the synthesis of conformationally restricted analogues of **1** and the findings from these studies will be presented in due course.

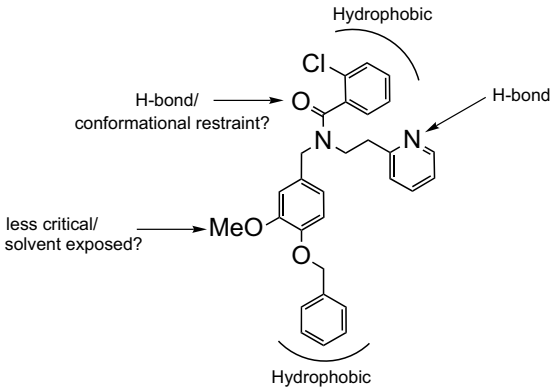


Figure 4. Structural features of **1** and analogues in relation to inhibition of the MAPK pathway based on the SAR study.

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19. Following 72 h serum starvation, HLR (HeLa Luciferase Reporter)-Elk1 cells (30,000 cells/well) were incubated for 1 h in the presence or absence of various concentrations of inhibitors prior to stimulation of Elk1 release by EGF addition (10 ng/mL). The amount of Elk1 released was measured after five hours using Bright-Glo Luciferase Assay (Promega Co., Madison, WI).
20. Proliferation assays were set up as follows: In a 96-well flat bottom plate, 5000 HCT-116 cells were plated per well and incubated overnight at 37 °C in 5% CO_2. The growth of plated cells was measured by adding 7.5 μM WST-1 reagent (Roche Applied Sciences, Germany) to three control wells and measuring OD_{650} and OD_{450} absorbances with a SpectraMax250 plate reader. If the OD_{650}–OD_{450} values were above 0.5, the remainder of the plate was used for incubation with compounds or solvent control for 48 h. After this incubation, WST-1 reagent was added to the wells and OD_{650}–OD_{450} values were calculated as before. Triplicate wells were assayed for each condition and standard deviation was determined; all experiments were performed at least three times independently.
21. (a) Soft agar growth assays were performed in a matrix consisting of a bottom agar (0.6%) and a top agar (0.3%) in RPMI media supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL penicillin, and 100 U/mL Streptomycin. A 24-well plate format was utilized with the top agar containing 1000 cells/well of HCT-116 cells. Cells were incubated in the presence or absence of various concentrations of compounds for a total of 10–20 days at 37 °C in a 5% CO_2 atmosphere. Colonies were stained with 0.2 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) overnight. Colony area was calculated and compared to DMSO treated cells, which served as the maximum colony formation control; (b) Finlin, B. S.; Gau, C.-L.; Murphy, G. A.; Shao, H.; Kimel, T.; Seitz, R. S.; Chiu, Y.-F.; Botstein, D.; Brown, P. O.; Der, C. J.; Tamanoi, F.; Andres, D. A.; Perou, C. M. *J. Biol. Chem.* **2001**, *276*, 42259.
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