Low PIAS3 expression in malignant mesothelioma is associated with increased STAT3 activation and poor patient survival

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ABSTRACT (word count: 248)

Purpose: Deregulation of STAT3 activation is a hallmark of many cancer cells and the underlying mechanisms are subject to intense investigation. We examined the extent of PIAS3 expression in mesothelioma cells and human tumor samples and determined the functional effects of PIAS3 expression on STAT3 signaling.

Experimental design: We evaluated the expression of PIAS3 in mesothelioma tumors from patients and correlated the expression levels with the course of the disease. We also measured the effects of enhanced PIAS3 activity on STAT3 signaling, cellular growth and viability in cultured mesothelioma cells.

Results: Gene expression databases revealed that mesotheliomas have the lowest levels of PIAS3 transcripts among solid tumors. PIAS3 expression in human mesothelioma tumors is significantly correlated with overall survival intervals (p = 0.058). The high expression of PIAS3 is predictive of a favorable prognosis and decreases the probability of death within one year after diagnosis by 44%. PIAS3 expression is functionally linked to STAT3 activation in mesothelioma cell lines. STAT3 down regulation with siRNA or enhanced expression of PIAS3 both inhibited mesothelioma cell growth and induced apoptosis. Mesothelioma cells are sensitive to curcumin and respond by the induction of PIAS3. Corroborative evidence has been obtained from STAT3 inhibition experiments. Exposure of the cells to a peptide derived from the PIAS3 protein which interferes with STAT3 function resulted in apoptosis induction and the inhibition of cell growth.

Conclusion: These results suggest that PIAS3 protein expression impacts survival in mesothelioma patients and that PIAS3 activation could become a therapeutic strategy.
TRANSLATIONAL RELEVANCE: The biological principle of negative feedback regulation is being exploited by tumor cells. Mesothelioma cells suppress PIAS3, a negative regulator of STAT3 activity. Strategies for the reactivation of PIAS3 could result in STAT3 inhibition and the growth arrest of tumor cells.

INTRODUCTION

The JAK-STAT signaling pathway provides an essential cellular communication route from the plasma membrane to the nucleus. It is initiated by the interaction of extracellular ligands with cellular surface receptors and results in alterations of transcription patterns. Cytokines and growth factors act as ligands and play central roles in the regulation of cell growth, differentiation and metabolism in many different cell types and organs (1). STAT proteins, especially STAT3, are not only important in the development and function of normal cells, but can assume oncogenic properties when the extent and duration of their activation are disturbed (2). Inappropriate STAT3 activation results in the prevention of apoptosis and the promotion of tumor cell proliferation (3, 4). STAT3 is constitutively phosphorylated and thereby activated in many forms of cancer. The inhibition of STAT3 activity often results in apoptosis and cell death (5, 6). STAT3 serves as a central integration point for multiple oncogenic signaling pathways to regulate genes involved in cell cycle control, apoptosis, angiogenesis, tumor invasion and metastasis. This makes it an attractive target for cancer therapies.

PIAS3 is part of a small gene family with four members (7, 8). PIAS3 was initially found to interact specifically with phosphorylated STAT3, and not STAT1, in IL-6-activated murine
myeloblast M1 cells and to decrease STAT3 DNA binding capacity and transcriptional activity (9). We (10, 11) and others (12) have demonstrated that overexpression of PIAS3 can inhibit STAT3 transcriptional activity and promote growth inhibition in vitro. If PIAS3 counteracts transactivation by STAT3, it would be conceivable that PIAS3 expression could be decreased in malignant tissues with high STAT3 activation. This hypothesis has been confirmed. PIAS3 expression is absent in glioblastoma multiforme (GBM) tumor tissue when compared to adjacent normal brain tissue (13). The expression of PIAS3 is inversely proportional to that of activated, phospho-STAT3 (pSTAT3) expression. We have examined expression of PIAS3 in 44 resected NSCLC specimens and found that 89% of adenocarcinomas stained positive for PIAS3 and only 38% of squamous cell carcinomas showed evidence of staining (14). In squamous cell carcinomas in which PIAS3 could be detected, only low expression levels were found. Intense PIAS3 expression in adenocarcinomas was associated with the absence of pSTAT3.

These studies suggest that PIAS3 may represent a useful target to devise therapeutic strategies against cancer cells harboring activated STAT3. Here we show that mesothelioma cell lines exhibit high STAT3 activity and use three experimental approaches to manipulate PIAS3 levels to demonstrate the principle. PIAS3 is able to inhibit STAT3 activity and cell growth in mesothelioma cells.

**MATERIAL AND METHODS**

**Cell culture and transient transfection**

Human pulmonary epithelial cell line A549 and mesothelioma cell lines H2052, H2452, 211H and H28 were purchased from American Type Culture Collection (Manassas, VA) and
maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, ThermoFisher Scientific, Waltham, MA) in a 5% CO₂ humidified incubator at 37°C. Cells were transfected with either pCMV5 (empty vector) or pCMV5-mouse PIAS3 using TransIT2020 (Mirus Bio LLC, Madison, WI). After 5 h, media was replaced with DMEM/F12 media containing FBS (10%). Following 24 h of incubation, cells were collected for further analysis.

**STAT3 siRNA transfection**

Down regulation of STAT3 by siRNA was achieved as described previously by Dabir et al (15).

**Immunoblotting and antibodies**

Whole cell lysates were prepared in RIPA buffer, as described previously (16). Antibodies used in western blots were obtained from either Cell Signaling Technology (PIAS3 #4641, pSTAT3 #9145, Caspase 3 #9961, PARP #9542) or Sigma (β-actin #A5441).

**Cell proliferation analysis**

Cell growth and viability were assessed in manually counted cells by either trypan blue dye exclusion or the MTS assay, as described previously (10).

**Purification and cell treatment of peptide rPP-C8**

Protein purification of the peptide rPP-C8 and cell treatment was performed as described previously (17). Briefly, two thousand cells were seeded in 96-well plates, and the next day medium was removed. 0.5 or 2.0 μM of the peptide (or PBS as solvent control) were diluted in 100 μL medium and added to the cells.
Immunohistochemistry

The TMA slides were obtained from the National Mesothelioma Virtual Bank (NMVB) (http://mesotissue.org/). The slides were deparaffinized with xylene rinses and then transferred through two changes of 100% ethanol. Endogenous peroxidase activity was blocked by 30 min incubation in a 2.5% hydrogen peroxide/methanol buffer. Antigen retrieval was performed by boiling the slides in a pressure cooker filled with a sodium citrate buffer (pH 6.0). After antigen retrieval, the slides were blocked using Background Sniper (Biocare #BS966M) for 20 min. The tissues were incubated with 1:400 dilution of rabbit anti-human PIAS3 antibody (Cell Signaling Technology) overnight at 4°C. Bound antibody was detected using an anti-rabbit MACH4 horseradish peroxidase-labeled polymer secondary antibody from Biocare (#MRH534L) for 30 min. The slides were rinsed in the TBS series, visualized with a 10-min incubation of liquid 3,3’-diaminobenzidine in buffered substrate in the dark. Finally, the slides were counterstained with hematoxylin for 30 min and mounted with Biomount. The intensity of immunostaining for PIAS3 was scored visually and stratified into 3 staining groups: 0 = no nuclear staining, 1+ = minimal staining and 2+ = moderate-to-strong staining in > 50% of tumor nuclei. At least two different cores were analyzed by IHC for ~70% of the tumors, ~50% had ≥ 3 cores analyzed per tumor. The multiple scores from cores of a single tumor specimen were averaged to yield the final score.

Statistical methods

Overall patient survival (OS) was measured from the date of diagnosis to the date of death. The difference of OS between groups was examined by log-rank test. The effect of PIAS3 as a continuous measurement was further evaluated using the Cox model (18) after controlling for
tumor grade (low, intermediate or high). Histologic type (epithelioid, sarcomatoid, biphasic) was not included in the Cox analysis due to a lack of some histotypes. The large majority of patients had advanced stage disease (stages III/IV) and therefore staging was also not included in the COX model used. The difference of PIAS3 expression between two groups was examined using a T-test. All tests are two-sided and a p-value ≤ 0.05 were considered statistically significant.

RESULTS

PIAS3 mRNA expression is low in cancer tissues

We assessed global gene expression patterns for PIAS3 expression across all types of cancer cell lines in the Cancer Cell Line Encyclopedia (CCLE) database (http://www.broadinstitute.org/ccle/home) (19). When cancer cell lines derived from solid tumors were compared, very low levels of PIAS3 were found in mesotheliomas (Figure 1A). When we specifically compared PIAS3 expression between non-small cell lung cancer (NSCLC) and mesothelioma cells, we found a significant difference in PIAS3 mRNA expression (Figure 1B, p <0.0007). Based on these observations, we further searched the Oncomine database (https://www.oncomine.org/resource/login.html) for analysis of PIAS3 expression in tumor tissues. The study by Gordon et al (20) compared PIAS3 expression in thoracic cancers. The results demonstrated that mesothelioma showed significantly lower expression of PIAS3 (p<0.001) compared to adenocarcinoma (Figure 1C).

PIAS3 expression predicts survival intervals of mesothelioma patients
We also investigated PIAS3 expression at the protein level. TMAs were obtained from the NMVB and PIAS3 expression analysis was performed by IHC. Representative pictures of PIAS3 staining and scoring for epithelioid cores are shown in Figure 2A. PIAS3 protein expression observed in the mesothelioma TMA showed mostly nuclear localization. The mean score for PIAS3 expression was $1.46 \pm 0.65$; and the median was 1.71. Thus, about half of the tissue cores demonstrated low or negative PIAS3 staining. These results demonstrated that PIAS3 expression in a subset of mesothelioma tumors was consistent with the mRNA expression results.

To determine whether PIAS3 expression levels are correlated with the survival intervals of patients upon diagnosis of the disease, we analyzed PIAS3 scoring of 39 epithelioid tumors with available patient survival information. We did not analyze other mesothelioma patients with biphasic, sarcomatoid and mixed histologies due to their low frequency. Kaplan-Meier survival analysis (21) showed that after controlling for the effects of tumor grade, PIAS3 expression significantly predicted OS intervals ($p = 0.058$). Mortality in patients with high PIAS3 expression was about 44% lower 12 months after diagnosis (Fig. 2B).

**STAT3 is activated in mesothelioma cell lines**

PIAS3 protein expression was also measured in four mesothelioma cell lines (H2052, H2452, 211H and H28) by Western blotting and compared to the expression levels in A549 lung cancer cells (Figure 3A), a representative NSCLC cell line (10, 11, 14, 15). Because PIAS3 is thought to be an endogenous inhibitor of STAT3 activity, we examined whether any decreased PIAS3 expression in mesothelioma cells was accompanied by increased p-STAT3 expression. The results in Figure 3A demonstrate an inverse correlation between PIAS3 and p-STAT3
expression; the three mesothelioma cells with high p-STAT3 levels (H2052, H2452, 211H) all have little detectable PIAS3 expression. The mesothelioma cell with high PIAS3 expression, H28, demonstrated no detectable p-STAT3. PIAS3 expression did not correlate with total STAT3 expression. Reprobing the blot with anti-β-actin shows that variability in protein loading could not account for the observed differences in p-STAT3 expression.

**STAT3 siRNA induces apoptosis in mesothelioma cells**

We established the functional significance of STAT3 activation in mesothelioma cells by measuring their survival and proliferation as a function of STAT3 transactivation. Down regulation experiments with STAT3 siRNA were carried out in H2052 and H2452 cell lines. Specific reduction in total STAT3 was demonstrated after STAT3 siRNA treatment, mismatch siRNA (mock) served as a negative control in H2052 and H2452 cells (Figure 3B). The reduction in STAT3 levels was accompanied by the induction of apoptosis, as shown by the appearance of cleaved caspase 3 and PARP. Further experiments demonstrated that knockdown of STAT3 with siRNA resulted in substantial growth inhibition in both mesothelioma cell lines, as measured by the MTS (Figure 3C) or by cell viability assays (Figure 3D). Taken together, these results demonstrate that STAT3 activity is required for mesothelioma cell growth and survival.

**PIAS3 overexpression inhibits cell growth and apoptosis in mesothelioma cells**
Most mesothelioma cells appear to be STAT3-dependent and we hypothesized that overexpression of PIAS3 would inhibit their growth. Indeed, transient overexpression of FLAG-tagged PIAS3 in H2052 and H2452 cells decreased p-STAT3 levels (Figure 4A). Empty vector (EV) transfected cells and untransfected cells served as controls (C). The decrease in activated STAT3 levels by PIAS3 overexpression also led to a decrease in cell growth, as measured by MTS (Figure 4B) or cell viability assays (Figure 4C). These results support our hypothesis that endogenous PIAS3 expression is downregulated in most mesothelioma cells and may thus contribute to the increased STAT3 activity that drives cell proliferation.

**Curcumin induces PIAS3 expression in MPM cell lines**

Enhancement of PIAS3 expression represents a potential therapeutic strategy for cancers like mesothelioma which depend on activated STAT3 for sustained growth. Thus, the discovery of a small molecule that increases endogenous PIAS3 levels in STAT3-dependent cancer cells could potentially become beneficial. Curcumin may be such a molecule, as it is a well-known inhibitor of STAT3 activity (22-24). Indeed, there is a time-dependent increase in PIAS3 levels in H2052 mesothelioma cells exposed to 1.0 µM curcumin (Figure 5A). This increase in PIAS3 was associated with a parallel decrease in p-STAT3 levels. No change in total STAT3 expression was observed, similar to β-actin. A dose-dependent increase in PIAS3 protein expression was observed upon curcumin treatment in all four mesothelioma cell lines (Figure 5B). Our experiments also demonstrated that curcumin treatment decreased mesothelioma cell growth (Figure 5C). These results suggest that curcumin may produce growth inhibition in mesothelioma cells through increased PIAS3 expression.
A recombinant PIAS3 peptide induces apoptosis and inhibits growth in mesothelioma cells

It has recently been shown that rPP-C8, a C-terminal recombinant peptide derived from PIAS3, has the ability to inhibit STAT3 activity and cell growth in glioblastoma cells (17). The peptide is able to penetrate the plasma membrane upon addition to the growth medium. We incubated this peptide with mesothelioma cells to determine its effect on STAT3 activity. Western blotting experiments showed a decrease in p-STAT3 levels in H2052 and 211H cells after a 4 h peptide treatment (Figure 6A). There was no effect on total STAT3 or PIAS3 expression levels. Furthermore, addition of rPP-C8 to the medium of mesothelioma cells induced apoptosis, as shown by increased PARP cleavage (Figure 6B). We next measured the effect of rPP-C8 on mesothelioma cell proliferation. H2052 and 211H cells were treated with 0.2 or 2.0 µM rPP-C8 peptide for 3 days. The mesothelioma cells demonstrated a dose-dependent decrease in cell viability upon addition of the peptide, compared to PBS-treated cells (Figure 6C). This was confirmed by cell counting (Figure 6D). Thus, the rPP-C8 peptide appears to be efficiently taken up by mesothelioma cells and acts as a potent STAT3 inhibitor in mesothelioma cell lines.

DISCUSSION

Our study addressed the question of whether the PIAS3 protein can be exploited as a potential drug target. We demonstrated elevated levels of STAT3 activation in mesothelioma and that mesothelioma may be ‘addicted’ to STAT3 activity using siRNA. A recent report also showed that the active forms of STAT3 are highly expressed in most cases of mesothelioma (25). Since the PIAS3 protein is being down regulated by cancer cells, a therapeutic strategy could be based
upon the enhancement of PIAS3 expression because of its role in inhibiting activated STAT3 and thereby inducing apoptosis. The number of cases of mesothelioma is increasing and current chemotherapy, radiotherapy and surgery treatments are mainly palliative (26). Clinical studies using molecular targeted approaches have been promising for NSCLC, like EGFR inhibition and angiogenic blockade, but have failed to improve patient outcome in mesothelioma patients (27, 28). Thus, new therapeutic strategies targeting PIAS3 could become valuable for cancers with persistent STAT3 activity, including mesotheliomas.

Many current cancer therapies target cell signal initiation at the level of membrane receptors, such as VEGFR and EGFR. However, multiple receptor tyrosine kinases are often activated in cancer, including mesothelioma (29, 30), making kinase specific inhibitors less effective or subject to the development of resistance. For example, two recent Phase II studies have shown that EGFR is highly expressed in 75-97% of mesothelioma tumor samples yet the disease was refractive to the EGFR tyrosine kinase inhibitors gefitinib (31) or erlotinib (32). These results indicate that downstream effectors of tyrosine kinase receptor signaling such as STATs may represent a more attractive choice for drug targeting because they represent an integration point for multiple oncogenic signaling pathways. Unfortunately, specific and effective STAT3 inhibitors are still only in early development, although promising lead compounds have recently been reported (33).

Here we report for the first time the clinical significance of PIAS3 expression on overall patient survival. In this regard, our study has provided preliminary data that PIAS3 expression may be useful as a prognostic factor in mesothelioma. Previous reports have used immunohistochemistry to suggest an important role for PIAS3 expression in subsets of gastrointestinal carcinomas, glioblastoma cell tumors and colorectal carcinoma (13, 34, 35). This
further emphasizes the potential role of PIAS3 targeted therapy in malignancies beyond mesothelioma.

Curcumin is regarded as the most active constituent derived from the spice turmeric (22). Turmeric has a long history in ancient Asian medicine and curcumin has been extensively studied in modern science as an anti-cancer agent both in vitro and in vivo. Here we show that curcumin treatment can increase PIAS3 levels and thereby decrease STAT3 phosphorylation and cell viability in mesothelioma cells. In support of our findings curcumin has been shown to increase PIAS3 levels and decrease STAT3 phosphorylation and cell viability in ovarian and endometrial cancer cells in vitro (24). These results suggest that curcumin may represent a lead compound to explore the potential of increasing endogenous PIAS3 levels as a therapeutic strategy against STAT3-dependent cancers. Although limited bioavailability of curcumin has hindered its clinical use (23), recent efforts to produce more pathway-specific analogs of curcumin, coupled with methods to increase its bioavailability, make this avenue more attractive for the treatment of mesothelioma (36, 37).

STAT3 inhibition can also be affected by peptides (5, 17, 38). Our study indicates that the uptake of a STAT3 inhibiting peptide into mesothelioma cells is effective and induces cell growth inhibition and apoptosis. There is an increase in the number of peptide drugs in preclinical and clinical development. Peptide degradation by proteolysis can be prevented by chemical modifications such as incorporation of D-amino acids or cyclization (39). Due to the tremendous advancement in the large scale synthesis of peptides it will be possible to make peptide-based anti-cancer drugs more affordable to patients (40). Thus, peptides are poised to make a huge impact in the near future in the area of cancer treatment and diagnosis.
In conclusion, here we present PIAS3 as a potential prognostic marker that would be beneficial in the management of mesothelioma due to the variable biological behavior of these tumors despite similar tumor stage, grade, and clinical presentations. Moreover, we present evidence that down regulation of STAT3 or induction of PIAS3 protein expression by curcumin or using a recombinant PIAS3 peptide in vitro affect the cell growth and induce apoptosis. Because the 3D crystal structure of PIAS3 has recently been solved (http://www.ebi.ac.uk/pdbe-srv/view/entry/4mvt/summary.html), this should hasten work on developing effective PIAS3 directed therapeutics using small molecule drug screens or finding structural analogs of the PIAS3 protein.

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FIGURE LEGENDS

Figure 1: Mesothelioma shows low PIAS3 expression in public datasets. (A) Global gene expression pattern for PIAS3 across all types of cancer cell lines in the Cancer Cell Line
Encyclopedia (CCLE) data base (B) PIAS3 expression in non-small cell lung cancer (NSCLC) and mesothelioma cells, showing a significant difference in PIAS3 mRNA expression in the CCLE database. (C) Whisker plots showing the Log2 median centered intensity of PIAS3 expression in Gordon lung. Compared to adenocarcinoma, PIAS3 expression in mesothelioma tissue samples is significantly lower.

Figure 2: Mesothelioma tumors demonstrate reduced PIAS3 expression. (A) Immunohistochemistry of mesothelioma tissue microarray: Representative pictures of PIAS3 staining and scoring. (B) Kaplan-Meier estimation of overall survival by PIAS3 level (epithelioid histology only, from Cox model).

Figure 3: Mesothelioma cells are dependent on high STAT3 activity for growth. (A) A549, H2052, H2452, 211H and H28 cells were grown under basal conditions and protein lysates immunoblotted for PIAS3, p-STAT3 (active), total STAT3 and β-actin. (B) H2052 and H2452 cells were treated with STAT3 siRNA, mismatch siRNA (mock) or untreated (control). Protein lysates were immunoblotted for total STAT3, cleaved caspase 3, PARP and β-actin. (C) Knockdown of STAT3 in mesothelioma cells promotes apoptosis (MTS assay) and (D) cell growth inhibition.

Figure 4: PIAS3 overexpression inhibits STAT3 activation and cell growth in mesothelioma cells. (A) H2052 and H2452 cells were transfected with FLAG-PIAS3 (TXN), empty vector (EV) or untreated (C); 48 h later whole cell extracts were prepared and immunoblotted for PIAS3, FLAG, pSTAT3 (active) and β-actin. (B) PIAS3 overexpression results in apoptosis (MTS assay) and (C) cell growth inhibition.
**Figure 5:** Curcumin increases endogenous PIAS3 expression in mesothelioma cells. (A) H2052 cell were incubated with 1µM curcumin for up to 24 h and protein lysates immunoblotted for PIAS3, pSTAT3 (active), total STAT3 and β-actin. (B) Curcumin treatment at 0.1 or 1.0 µM for 24 h shows an increase in PIAS3 protein expression level in most of the mesothelioma cell lines. (C) Curcumin inhibits cell proliferation. H2052 and H2452 cells were incubated for 24 h in the absence or presence of 10 µM of curcumin and cell growth determined by DNA assay.

**Figure 6:** A recombinant PIAS3 peptide inhibits STAT3 activation and cell growth in mesothelioma cells. (A) Protein lysates were prepared after 4 h exposure of H2052 and 211H cell lines to rPP-C8 peptide (0.5 and 2.0 µM). Western blotting shows decrease in pSTAT3 (Tyr 705) without affecting total STAT3 or PIAS3 expression. β-actin is a loading control. (B) H2052 and 211H cells exposed to 0.5 or 2.0 µM rPCC8 peptide for 24 h results in PARP cleavage at the higher concentration. (C) Addition of the peptide to mesothelioma cells induces apoptosis (MTS assay) and (D) cell growth inhibition.

**REFERENCES**


Figure 2

(A) Images of tissue sections stained for PIAS3.

(B) Kaplan-Meier survival curve showing the probability of overall survival for PIAS3 < 2 (n = 23) and PIAS3 = 2 (n = 16). The p-value is 0.05.

Probability of overall survival

0 6 12 18 24

0
20
40
60
80
100

PIAS3 < 2 (n = 23)
PIAS3 = 2 (n = 16)

p-value = 0.05

Months after diagnosis
Figure 3

A

A549  H2052  H2452  211H  H28

PIAS3  p-STAT3  t-STAT3  β-Actin

B

Control  Mock  STAT3  Control  Mock  STAT3

siRNA

siRNA

t-STAT3  Cleaved Caspase3  Cleaved PARP  β-Actin

C

Absorbance at 490 nm

Control  Mock  siRNA-STAT3 Txxn

H2052

D

Cell Count x10^3

Day

H2052

H2452

siRNA  Mock  Control

siRNA  Mock  Control

0  1  2  3
Figure 4

A

![Western Blot Images]

- PIAS3
- FLAG
- p-STAT3
- β-Actin

H2052  H2452

B

**Absorption at 490 nm**

- Control
- Mock
- PIAS3 Txn

Control  Mock  PIAS3 Txn

C

**Cell Count x 10^3**

- H2052
- H2452

- PIAS3 txn
- Mock
- Control

Day 1  2  3
Figure 5

A

PIAS3

p-STAT3

t-STAT3

β-Actin

hrs

0 ½ 2 4 8 24

B

C

Relative cell growth (%)

Con Treated Con Treated

H2052 H2452

Curcumin (µM)

0.1 1.0

PIAS3

β-Actin

H2052 H2452 H28 211H

Curcumin (µM)

0.1 1.0

PIAS3

β-Actin

H2052 H2452 H28 211H
Figure 6

A  
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H2052  211H

B  
| + C | C | 0.5 | 2.0 | C | 0.5 | 2.0 (µM) |
|---|---|---|---|---|---|
| rPP-C8 (µM) |
| PARP Cleavage |
| β-Actin |

H2052  211H

C  

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H2052  211H

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H2052  211H
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