LETTER

Selective killing of cancer cells by a small molecule targeting the stress response to ROS

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Malignant transformation, driven by gain-of-function mutations in oncogenes and loss-of-function mutations in tumour suppressor genes, results in cell deregulation that is frequently associated with enhanced cellular stress (for example, oxidative, replicative, metabolic and proteotoxic stress, and DNA damage)[1]. Adaptation to this stress phenotype is required for cancer cells to survive, and consequently cancer cells may become dependent upon non-oncogenes that do not ordinarily perform such a vital function in normal cells. Thus, targeting these non-oncogene dependencies in the context of a transformed genotype may result in a synthetic lethal interaction and the selective death of cancer cells[2]. Here we used a cell-based small-molecule screening and quantitative proteomics approach that resulted in the unbiased identification of a small molecule that selectively kills cancer cells but not normal cells. Piperlongumine increases the level of reactive oxygen species (ROS) and apoptotic cell death in both cancer cells and normal cells engineered to have a cancer genotype, irrespective of p53 status, but it has little effect on either rapidly or slowly dividing primary normal cells. Significant antitumour effects are observed in piperlongumine-treated mouse xenograft tumour models, with no apparent toxicity in normal mice. Moreover, piperlongumine potently inhibits the growth of spontaneously formed malignant breast tumours and their associated metastases in mice. Our results demonstrate the ability of a small molecule to induce apoptosis selectively in cells that have a cancer genotype, by targeting a non-oncogene co-dependency acquired through the expression of the cancer genotype in response to transformation-induced oxidative stress[3–5].

Using a luciferase reporter gene fused with the *CDIP* (cell death involved p53 target, also known as 5730403B10Rik) promoter[6], we performed a small-molecule screen (Supplementary Fig. 1) to identify compounds acting through novel pro-apoptotic mechanisms. The compound with the highest composite Z value was piperlongumine (Supplementary Fig. 2a), which increased luciferase activity from the reporter gene at levels comparable to the positive control, etoposide (Supplementary Figs 2b and 3). Piperlongumine is a natural product isolated from the plant species *Piper longum* L. (Fig. 1a) and it was previously shown to have cytotoxic effects[7]. We examined the effects of piperlongumine on the viability of cultured cancer cells and normal cells (Fig. 1b and Supplementary Figs 4 and 6). Piperlongumine treatment markedly induced cell death in cancer cells with both wild-type p53 and mutant p53. When primary normal cells and non-transformed immortalized cells with diverse proliferative capacities were incubated with highly purified piperlongumine (Supplementary Fig. 5) for 24 h (under the indicated conditions, which avoid spontaneous transformation and minimize stress), there was little apparent reduction in cell viability, even at the highest concentration tested (15 μM, a concentration of piperlongumine that approaches its solubility limit). This indicated that piperlongumine may have a cancer-cell-selective killing property, and that sensitivity to piperlongumine may result from the process of malignant transformation. To test this hypothesis, we used a

defined model[8] of oncogenic conversion of normal cells through ectopic expression of the telomerase catalytic subunit (*hTERT*) in combination with small T antigen and an oncogenic allele of *HRAS* (Fig. 1c), and
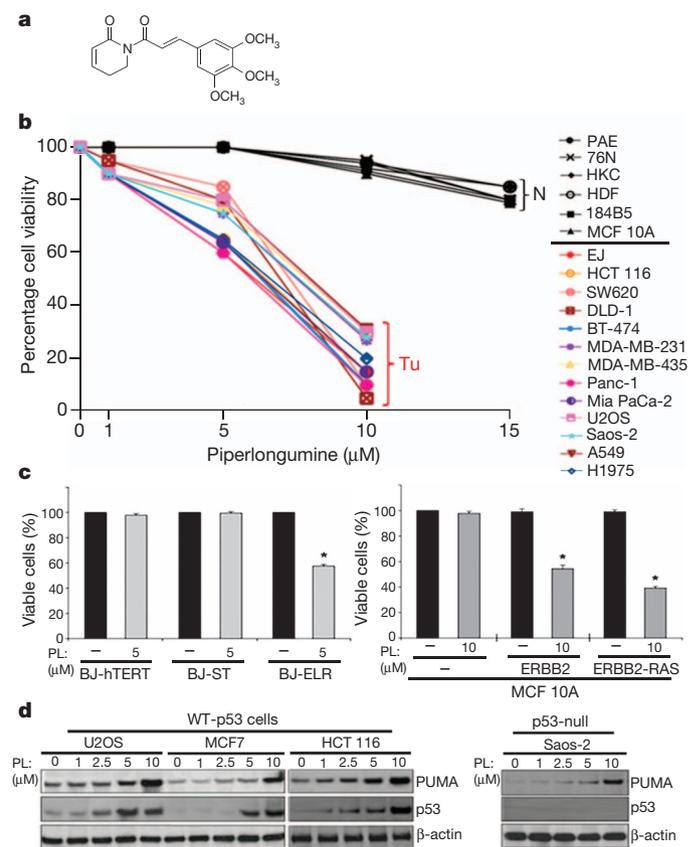

Figure 1 | Selective killing effect of piperlongumine in cancer cells.
a, Structure of piperlongumine. **b**, Piperlongumine treatment induces cell death in cancer cells but not in normal cells. Normal human cells (N), including aortic endothelial cells (PAE), breast epithelial cells (76N), keratinocytes (HKC) and skin fibroblasts (HDF), as well as two immortalized breast epithelial cell lines (184B5 and MCF 10A), were grown in 12-well or 24-well plates and treated with piperlongumine at 1–15 μM for 24 h. A variety of human cancer cell lines (Tu) were also treated with piperlongumine or DMSO (control) for 24 h. Cytotoxicity was measured by trypan blue exclusion staining (average of three independent experiments). Piperlongumine was HPLC-purified (~99% purity) before the treatment. **c**, Selective cell death caused by piperlongumine (PL) in oncogenically transformed human BJ skin fibroblasts (left panel) and MCF 10A cell lines (right panel). A representative graph for cell viability is shown (mean ± s.d. of three independent experiments; *, $P < 0.0001$). **d**, The effects of piperlongumine on p53 and its target PUMA were measured by western blot analyses in several cancer cell lines. β-actin expression was used as a loading control.

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observed sensitivity to piperlongumine upon oncogenic transformation of normal cells. Similar results were obtained using serial transformation of spontaneously immortalized MCF 10A breast epithelial cells by overexpression of *ERBB2* and/or *HRAS*[9] (Fig. 1c).

Western blot analysis showed that wild-type p53 expression was significantly enhanced in different types of cancer cells by treatment with piperlongumine (Fig. 1d). Moreover, a p53 proapoptotic target, BCL2 binding component 3 (BBC3, also known as PUMA), was significantly induced in response to piperlongumine, even in p53-null Saos-2 cancer cells (Fig. 1d). Piperlongumine treatment was able to repress the expression of several pro-survival proteins, including B-cell CLL/lymphoma 2 (BCL2), baculoviral IAP repeat containing 5 (also known as survivin) and X-linked inhibitor of apoptosis (XIAP) (Supplementary Fig. 7). Among 55 death- or survival-related genes, we observed increased levels of apoptotic transcripts and decreased levels of pro-survival transcripts in cancer cells in the presence of piperlongumine, but no significant changes in these transcripts in normal cells (Supplementary Fig. 8). These results indicate that piperlongumine induces cell death or apoptosis (Supplementary Fig. 4a, e) preferentially in cancer cells by modulating the expression of members of apoptotic and survival pathways, including p53 targets and p53 itself, and that it does not require p53 for this activity.

We next tested piperlongumine in established tumour xenografts in mice (human bladder, breast and lung tumours in nude mice, and mouse melanoma in C57BL/6 mice; Supplementary Fig. 9). Marked antitumour effects were observed in tumour-bearing mice treated with piperlongumine, as compared to dimethyl sulphoxide (DMSO)-treated controls (Supplementary Fig. 9). Piperlongumine treatment enhanced the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A, or p21$^{WAF1/CIP}$), PUMA and caspase 3 in EJ-cell tumours (Supplementary Fig. 10a). Moreover, piperlongumine treatment inhibited the formation of blood vessels in xenograft-tumour mice (Supplementary Figs 9d and 10b). We also studied piperlongumine in a transgenic mouse model of spontaneous breast cancer, MMTV-PyVT[10]. When tumour sizes had grown to about 5–6 mm in diameter (in female MMTV-PyVT mice, 8–9 weeks of age), piperlongumine was administered intraperitoneally (2.4 mg kg^{-1}) daily for two weeks and notable antitumour effects were observed (Fig. 2a, b). Furthermore, there were no secondary tumours in piperlongumine-treated mice compared to vehicle-treated controls. At day 13, the vehicle-treated control mice showed severe malignant progression indicated by the formation of aggressive adenocarcinoma (Fig. 2c). In contrast, the mammary glands of piperlongumine-treated mice were preserved and the tissue showed a hyperplastic-like, non-malignant phenotype (Fig. 2c). Notably, piperlongumine seemed to be more effective in tumour growth inhibition than paclitaxel (Fig. 2d). Piperlongumine also showed excellent oral bioavailability and desirable exposure levels (C_{max} and bioavailability, measured by calculating the area under curve (AUC)) in mice, as observed after a single oral administration and after intravenous injection (Supplementary Fig. 11). To examine potential cytotoxic side-effects of piperlongumine on normal tissues, CD-1 mice were intraperitoneally treated with piperlongumine (2.4 mg kg^{-1}) or DMSO, daily for 6 days, and whole blood samples as well as vital organs were collected for haematology and histopathological analyses, respectively. Piperlongumine-treated CD-1 mice remained healthy throughout the treatment time and no notable differences between the vehicle-treated and piperlongumine-treated groups were evident (Supplementary Figs 12 and 13). High-dose acute toxicity studies demonstrated that piperlongumine did not cause any obvious clinical indications (Supplementary Fig. 14 and Supplementary Tables 1 and 2). Together, these results indicate that treatment with piperlongumine potently suppresses tumour growth in diverse tissues without affecting normal tissues in mice.

We next used a method combining affinity enrichment with stable-isotope labelling with amino acids in cell culture (SILAC) and quantitative proteomics to identify the target proteins and their associated
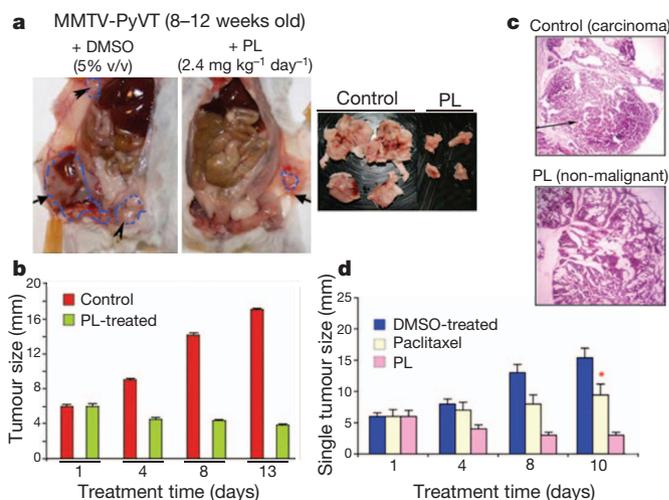

Figure 2 | *In vivo* antitumour effect of piperlongumine. a, Inhibition of mammary tumour growth by piperlongumine treatment in MMTV-PyVT transgenic tumour-bearing mice. MMTV-PyVT mice spontaneously developed breast adenocarcinoma by ~8 weeks of age. When tumours had grown to ~5–6 mm in diameter, piperlongumine (2.4 mg kg^{-1}), paclitaxel (10 mg kg^{-1}) or DMSO (5% v/v) was administered intraperitoneally daily for 13 days ($n = 12$ mice per group). Mice were then euthanized and mammary tumours were excised and processed for histological examination. Arrows indicate the initial as well as secondary tumour lesions in the DMSO treated animals (left panel) versus only a single initial lesion in the piperlongumine treated animals (right panel). **b**, The sizes of the grossly dissected tumours were measured and plotted. **c**, Histological morphology of mammary tissue sections from MMTV-PyVT tumour-bearing mice treated with piperlongumine or DMSO after 13 days, stained with haematoxylin and eosin. **d**, Size of single tumours after 10 days of piperlongumine or paclitaxel treatment (asterisk indicates shorter treatment period due to high toxicity of paclitaxel in animals). Values in bar graphs are mean ± s.d. of three independent experiments.

complexes that bind to piperlongumine[11] (Supplementary Figs 15 and 16a and Supplementary Methods). Twelve interaction partners of piperlongumine that were similar in both EJ and U2OS cells were identified (Supplementary Fig. 16b). Seven of these, including the top four high-signal outliers, are known to participate in the cellular response to oxidative stress caused by elevated ROS. Glutathione *S*-transferase pi 1 (GSTP1) was the highest-confidence hit, followed by carbonyl reductase 1 (CBR1) (Supplementary Fig. 16b). Several of these proteins are known to be part of a common complex[12,13], indicating that the affinity purification may have identified direct and indirect partners.

These results indicate that, by binding to proteins known to regulate oxidative stress, piperlongumine may modulate redox and ROS homeostasis. Consistent with this hypothesis, we found that piperlongumine can interact directly with purified recombinant GSTP1 and inhibit its activity (Supplementary Figs 17 and 18), and also that it can lead to a decrease in reduced glutathione (GSH) levels and an increase in oxidized glutathione (GSSG) levels in cancer cells (Fig. 3a). Piperlongumine treatment did not increase GSSG levels in normal cells (76N (NMEC)) (Fig. 3a). Furthermore, co-treatment with piperlongumine and the reducing agent *N*-acetyl-L-cysteine (NAC, 3 mM), which quenches ROS, prevented piperlongumine-mediated GSH depletion (Fig. 3a).

We next determined the effect of piperlongumine on cellular ROS levels in several human cancer cells (EJ, MDA-MB-231, U2OS and MDA-MB-435) through flow cytometry using the redox-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA). Treatment with piperlongumine for 1 h and 3 h caused a marked increase in ROS levels in these cancer cells (Fig. 3b and Supplementary Figs 19 and 20). Paclitaxel also caused an increase in DCF-DA fluorescence after 1 h, but piperlongumine enhanced ROS to nearly
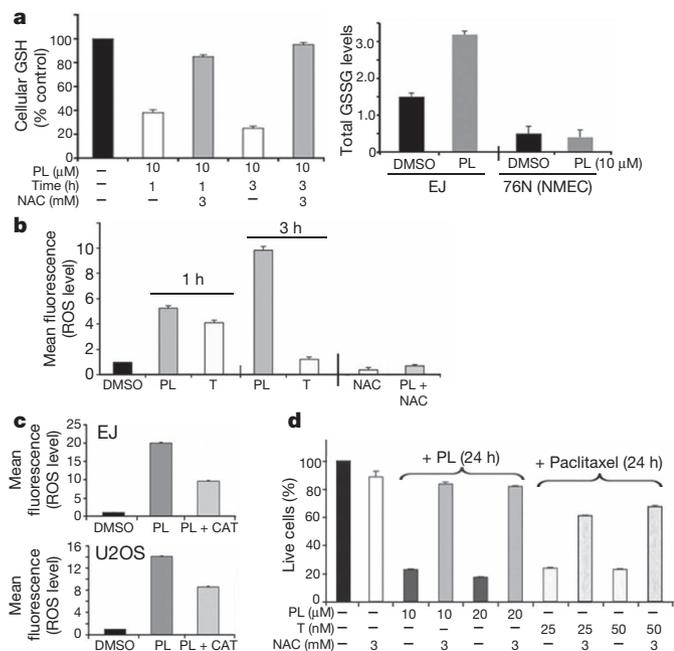
Figure 3 | Piperlongumine enhances ROS accumulation in cancer cells by targeting the stress response to ROS. a, Piperlongumine-mediated modulation of GSH and GSSG. GSH levels were determined after EJ cells were either treated with piperlongumine or pretreated with NAC for 1 h, followed by piperlongumine treatment for 1 h or 3 h (left panel). GSSG levels were also determined after EJ cells and 76N (NMEC) cells were treated with piperlongumine for 3 h (right panel). **b**, Piperlongumine-induced ROS elevation and reversion by NAC. EJ cells were treated with piperlongumine (PL, 10 μM), paclitaxel (T, 25 nM) or DMSO for 1 h and 3 h. Cells were also pretreated with 3 mM NAC for 1 h, followed by 10 μM piperlongumine for 3 h. **c**, Reversion of piperlongumine-induced ROS accumulation by catalase. EJ or U2OS cells were pretreated with catalase (CAT, 2,000 U ml^{-1}) for 2 h, followed by 10 μM piperlongumine for 3 h. **d**, Piperlongumine-induced cell death can be rescued by NAC. EJ cells were treated with piperlongumine for 24 h, or treated with 3 mM NAC for 1 h followed by piperlongumine or paclitaxel for 24 h. Cell viability was measured by trypan blue exclusion staining assay. All values are mean ± s.d. of three independent experiments.

twice these levels (Fig. 3b). Co-treatment with NAC fully reversed the piperlongumine-induced increase in ROS and cell death (Fig. 3b, d and Supplementary Fig. 21). Using a series of fluorescent probes specific for individual species of ROS, we found that hydrogen peroxide and nitric oxide, but not superoxide anion, were among the ROS species induced by piperlongumine in cancer cells (Fig. 3b, c and Supplementary Figs 22–24).

In contrast to the results in cancer cells, piperlongumine did not cause an increase in ROS levels in normal cells (Fig. 4a and Supplementary Fig. 25). This selective induction of ROS in cancer cells distinguishes piperlongumine from other small molecules that affect ROS levels, such as the microtubule-stabilizing agent paclitaxel and the glutathione synthesis inhibitor buthionine sulphoximine (Fig. 4a and Supplementary Fig. 25), and indicates that piperlongumine-induced ROS elevation is a consequence of cell transformation. Engineering normal cells to have a cancer genotype potentiated the piperlongumine-induced increase in ROS (Fig. 4b and Supplementary Figs 26 and 27). Serial transformation itself leads to increased expression of the putative piperlongumine targets GSTP1 and CBR1 (Supplementary Fig. 28), indicating that these proteins may have a role in enabling the transformed cell to adapt to transformation-induced oxidative stress. We therefore hypothesized that overexpression of CBR1 or GSTP1 might rescue transformed cells from both piperlongumine-induced ROS elevation and piperlongumine-induced apoptosis. Stably overexpressing CBR1 or GSTP1, and particularly both, in EJ cells markedly reduced piperlongumine-induced ROS levels and partially rescued
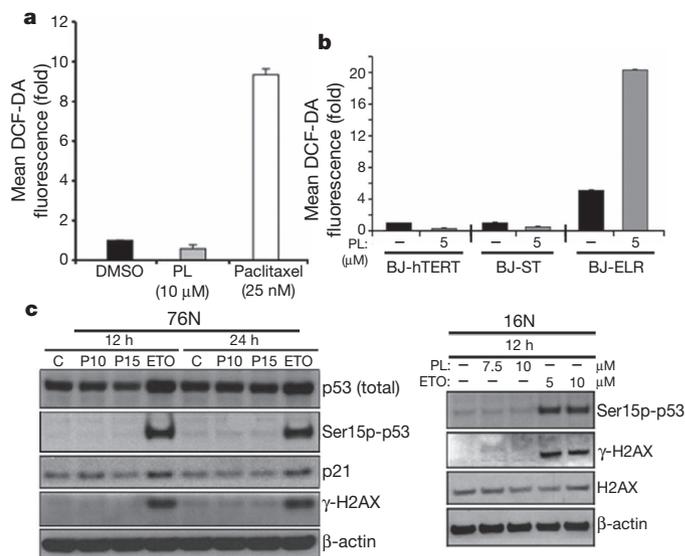

Figure 4 | Piperlongumine does not increase ROS levels and the ROS-induced DNA-damage response in normal and immortalized non-transformed cells. a, Piperlongumine does not increase ROS levels in normal cells (16N). ROS levels were measured by flow cytometry and shown by quantitative bar graph measured as the fold change over DMSO-treated levels. **b**, Selective induction of ROS by piperlongumine in oncogenically transformed BJ human fibroblasts (BJ-ELR), but not in non-transformed BJ fibroblasts (BJ-hTERT and BJ-ST). ROS levels were measured after treating with DMSO (−) or piperlongumine (PL, 5 μM) for 8 h. All values are mean ± s.d. of three independent experiments. **c**, The effect of piperlongumine on stress-response targets was determined by western blot analysis of p53, phosphorylated p53 (Ser 15 p-p53), p21 and γ-H2AX in normal 76N cells, treated with piperlongumine (10 μM (P10) or 15 μM (P15)), 10 μM etoposide (ETO) or DMSO as a solvent control (C) for 12 h and 24 h (left panel). Western blots were performed similarly on 16N cells treated for 12 h with piperlongumine (7.5 μM or 10 μM) or etoposide (5 μM or 10 μM) (right panel). β-actin expression was used as a loading control.

the piperlongumine-induced apoptotic phenotype (Supplementary Fig. 29). In a complementary study, knockdown of GSTP1 or CBR1 did not affect piperlongumine-induced ROS levels (Supplementary Fig. 30). These results may reflect the fact that other members of the GST family were observed to bind piperlongumine in our affinity-enrichment studies (Supplementary Fig. 16b) and may have partially overlapping functions in the cell. These data indicate that piperlongumine induces apoptosis by interfering with redox and ROS homeostatic regulators such as GSTP1 and CBR1.

The ability of piperlongumine to inhibit the growth of rapidly growing and highly invasive multifocal mammary tumours without general toxicity indicates that perturbing redox and ROS homeostasis is a promising strategy for cancer treatment. Our cell-based experiments indicate that piperlongumine treatment selectively increases ROS levels and induces apoptosis in cancer cells relative to normal cells. This correlates with the selective induction of related phenotypes, including DNA damage (Fig. 4c and Supplementary Figs 27, 31 and 32) and alterations in mitochondrial morphology and function, occurring selectively in cancer cells (Supplementary Fig. 33). The differential response of cancer cells and normal cells to treatment with piperlongumine indicates that piperlongumine targets a dependency associated with ROS homeostasis that arises during transformation. Normal cells, including stem cells, have low basal levels of ROS[1,4,5,14–17] and therefore a diminished reliance on the ROS stress-response pathway, whereas cancer cells, especially cancer stem cells, have high levels of ROS[14] and might therefore be expected to have a strong reliance on the ROS stress-response pathway[1,5,16,18,19]. The use of small molecules that alter levels of ROS such as β-phenylethyl-isothiocyanate and buthionine sulphoximine[4,17] has been suggested for the treatment of cancer. Other small molecules such as curcumin[20] and 2-cyano-3, 12-dioxoolean-1,9-dien-28-oic acid (CDDO) derivatives[21] have been

reported to promote ROS and reduce GSH levels in cancer cells, in one case in an oncogene-dependent manner[17], and the activation of the KEAP1–NRF2 antioxidant pathway[22,23] has been suggested to be involved.

The introduction of a single oncogene (*HRAS*) leads to increased levels of ROS (Fig. 4b and ref. 24), increased expression of GSTP1 and CBR1 (Supplementary Fig. 28), an increased apoptotic response to piperlongumine (Fig. 1c), and notably, to a substantial increase in levels of ROS after treatment with piperlongumine. In EJ cells, piperlongumine-induced cell death is rescued by the antioxidant NAC (Fig. 3d). The increased dependence of cancer cells on the ROS stress-response pathway may be the basis for the selectivity of piperlongumine-induced apoptosis in cancer cells (Figs 1 and 2). In support of this hypothesis, the activation of signalling through the JNK (also known as MAPK8) pathway has been implicated as an antitumorigenic response to oncogene expression[25]. This response is coupled to oncogene-dependent oxidative stress through p53 stabilization, and could also function independently of p53 through pro-apoptotic cJUN-dependent transcription. In addition to its role in regulating ROS, GSTP1 is also known to be a direct negative regulator of JNK[26], providing a possible mechanism for piperlongumine-induced apoptosis in both p53-wild-type and p53-mutant cancer cells.

A global investigation of the spectrum of cancer genotypes will be required to identify the range of cancer genotypes that impart piperlongumine sensitivity, but our results already highlight a novel strategy for cancer therapy that preferentially eradicates cancer cells by targeting the ROS stress-response pathway.

METHODS SUMMARY

Apoptotic cell populations were determined by TdT-mediated dUTP nick end labelling (TUNEL) assay and quantified using flow cytometry. Cell viability was also determined by crystal violet staining (0.2% w/v in 2% ethanol), by trypan blue exclusion and by the Alamar blue cell viability assay. For crystal violet staining, cells were plated in 6-well and 12-well plates and, after reaching 60–70% confluency, the cells were treated with piperlongumine for 12 h and 24 h. For measurement of ROS production, cells were treated with piperlongumine or paclitaxel for 1 h and 3 h and then incubated with 10 μM DCF-DA for 30 min at 37 °C, washed twice with PBS and immediately analysed by a FACScan flow cytometer. Cells were treated with piperlongumine and etoposide for 18–24 h and processed for Comet assay following the manufacturer's instructions (Trevigen). For xenograft tumour models, cancer cell lines EJ, A549 and MDA-MB-435 were injected subcutaneously into the flanks of nude mice. For the melanoma mouse model, B16-F10 melanoma cells were injected into the flanks of C57BL/6J mice. FVB/N-Tg (MMTV-PyVT) 634Mul males were obtained from the Mouse Models of Human Cancer consortium (MMHCC) at NCI-Frederick and bred with FVB females. Female offspring were genotyped for the presence of the transgene using the primers published by MMHCC. For piperlongumine target identification, we followed the SILAC-based affinity enrichment methodology previously described[11,27]. For further details see Supplementary Methods.

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Author Contributions L.R. and T.I. conducted most of the experimental work. A.U.G., M.S. and X.L. made critical experimental contributions. N.J.T., A.M.S., T.R.G., S.A.C., A.F.S. and M.F. designed the experimental plans, analysed and interpreted the data. A.M., S.L.S. and S.W.L. designed and directed the project and drafted the manuscript.