A phospho-proteomic study of cetuximab resistance in \textit{KRAS/NRAS/BRAF}^{V600} wild-type colorectal cancer

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Abstract

\textbf{Purpose} We hypothesised that plasticity in signal transduction may be a mechanism of drug resistance and tested this hypothesis in the setting of cetuximab resistance in patients with \textit{KRAS/NRAS/BRAF}^{V600} wild-type colorectal cancer (CRC).

\textbf{Methods} A multiplex antibody-based platform was used to study simultaneous changes in signal transduction of 55 phospho-proteins in 12 \textit{KRAS/NRAS/BRAF}^{V600} wild-type CRC cell lines (6 cetuximab sensitive versus 6 cetuximab resistant) following 1 and 4 h in vitro cetuximab exposure. We validated our results in CRC patient samples (n = 4) using ex vivo exposure to cetuximab in \textit{KRAS/NRAS/BRAF}^{V600} cells that were immunomagnetically separated from the serous effusions of patients with known cetuximab resistance.

\textbf{Results} Differences in levels of phospho-proteins in cetuximab sensitive and resistant cell lines included reductions in phospho-RPS6 and phospho-PRAS40 in cetuximab sensitive, but not cetuximab resistant cell lines at 1 and 4 h, respectively. In addition, phospho-AKT levels were found to be elevated in 3/4 patient samples following ex vivo incubation with cetuximab for 1 h. We further explored these findings by studying the effects of combinations of cetuximab and two PI3K pathway inhibitors in 3 cetuximab resistant cell lines. The addition of PI3K pathway inhibitors to cetuximab led to a significantly higher reduction in colony formation capacity compared to cetuximab alone.

\textbf{Conclusion} Our findings suggest activation of the PI3K pathway as a mechanism of cetuximab resistance in \textit{KRAS/NRAS/BRAF}^{V600} wild-type CRC.

Keywords Colorectal cancer · Cetuximab · Proteomics · Phospho-proteomics · Signalling adaptations · Resistance mechanisms

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\textbf{Abbreviations}

AKT Serine-threonine protein kinase also called protein kinase B

ALK Anaplastic lymphoma kinase

BRAF V-raf murine sarcoma viral oncogene homolog B1

CCR7 C–C chemokine receptor type 7

CHK Checkpoint kinase

Cmax The peak serum concentration

EGFR Epidermal growth factor receptor

ErbB Family of proteins—contains four receptor tyrosine kinases, structurally related to the epidermal growth factor receptor

GI50 Growth inhibitory 50%

HER2 Human epidermal growth factor receptor 2

HER3 Human epidermal growth factor receptor 3

ICR Institute of Cancer Research London UK

JAK Janus Kinase
KRAS Kirsten rat sarcoma viral oncogene  
MAPK Mitogen-activated protein kinases  
MEK Mitogen-activated protein kinase kinase  
MET MET proto-oncogene, receptor tyrosine kinase  
MFI Median fluorescent intensity  
mTOR Mammalian target of rapamycin  
NRAS Neuroblastoma ras viral oncogene homologue  
NTRK Neurotrophic-receptor tyrosine kinase  
PDGF Platelet growth factor receptor  
PI3K Phosphatidylinositol 3-kinase  
PIK3CA Phosphatidylinositol 3-kinase catalytic alpha polypeptide  
PRAS40 He proline-rich Akt substrate of 40 kDa  
ROS Proto-oncogene tyrosine-protein kinase ROS  
SD Standard deviation  
SRB Sulforhodamine B  
SRC Proto-oncogene tyrosine-protein kinase Src  
STAT Signal transducer and activator of transcription protein  
WT Wild-type

1 Introduction

Cetuximab is a monoclonal antibody that targets the epidermal growth factor receptor (EGFR). As a single agent and in combination with cytotoxic agents, cetuximab has been shown to improve overall survival, when compared to placebo or standard of care, in patients with metastatic KRAS and NRAS exon 2–4 wild-type (WT) colorectal cancer (CRC) [1–3]. However, even in patients with KRAS WT tumours, with resistance to standard of care chemotherapy, the clinical efficacy of cetuximab is modest, with a monotherapy radiological response rate of 20% [1]. Evolutionary changes leading to the selection of the “fittest” RAS mutant resistant clones and other genetic aberrations such as PIK3CA, BRAF and extracellular EGFR domain mutations, HER2 amplifications and ALK/ROS1/NTRK/RET fusions have also been suggested as mechanisms of anti-EGFR antibody resistance [4–7]. In addition to genetic aberrations, compensatory signalling crosstalk (for example between EGFR and MET receptor or EGFR with other ErbB family receptors) have been suggested to contribute to the development of cetuximab resistance [6, 8].

Phospho-proteomic changes following exposure to targeted therapies can be regarded as a quantifiable surrogate of adaptive signalling. To date, no studies have been reported on the profiling of phospho-proteomic changes upon exposure to cetuximab in KRAS/NRAS/BRAFV600 WT CRC cells. We hypothesised that studying re-wiring of signal transduction in response to cetuximab may provide novel insights into mechanisms of drug resistance in the setting of RAS/BRAFV600 WT CRC. To test this hypothesis, we utilised 12 KRAS/NRAS/BRAFV600 WT cell lines, representing both cetuximab sensitive and resistant cells, and patient-derived samples that were isolated from serous effusions of patients with KRAS/NRAS/BRAFV600 WT CRC, who were resistant to cetuximab. We next used a multiplex antibody-based proteomic platform to quantify simultaneous changes in 55 phospho-proteins, following 1 and 4 h of cetuximab exposure. Our phospho-proteomic panel included 26 receptor and non-receptor tyrosine kinases as well as phospho-proteins that function within pathways that are known to be key in CRC, including the MAPK, PI3K, JAK-STAT and Wnt/β-Catenin pathways. All findings of interest were validated and further investigated.

2 Methods

2.1 Cell lines, tissue culture and anti-cancer agents

The SNUC1, CACO2 and LIM1215 cell lines were purchased from the American Tissue Culture Collection (ATCC) and the C10, C70 and HCA24 cell lines from Public Health England (PHE). The NCIH508, HCA46, SW48 and COLO320 cell lines were kindly donated by other laboratories within the Institute of Cancer Research (ICR) London. OXCO2 and DiFi were kindly donated by Professor Alberto Bardelli’s team, Candiolo Cancer Institute, Turin, Italy. The known mutations in the cell line panel are described in Supplementary Table 1. Cetuximab was kindly provided by Merck Serono Ltd., UK, an affiliate of Merck KGaA, Darmstadt, Germany. GDC0941 and AZD2014 were purchased from Selleck Chemicals.

2.2 Isolation of cancer cells from serous effusions of patients with KRAS/NRAS/BRAFV600 WT CRC

Up to 1000 ml ascites or pleural fluid was collected from patients at the time of therapeutic drainage after which cancer cells were immunomagnetically separated on the same day using previously published methods [9, 10].

2.3 Quantification of phosphoproteins

2.3.1 Lumines 200 magnetic bead suspension array

The material and methods used have been described previously [9]. All phospho-proteins that were quantified are listed in Supplementary Fig. 1. In brief, cells were equally seeded in 25 cm² flasks and cultured to 70% confluency. Next, the cells were exposed to cetuximab or left untreated for 1 and 4 h. To closely model the clinical setting, the concentration of cetuximab used (306 µg/ml) was equal to the peak serum concentration (Cmax) that cetuximab has been
reported to reach in patients’ plasma in early phase clinical trials [11]. Since cetuximab protein binding in plasma is negligible, the Cmax concentration in cell culture was not modified [12]. After 1 or 4 h exposure, cells were lysed in 43–040 (MerckMillipore) supplemented with protease (I3786, Sigma Aldrich, UK) and phosphatase (4,906,845,001, Sigma Aldrich) inhibitors. Ten μg protein was loaded in each well of a 96-well plate and processed as per manufacturers’ protocol. The quality control measures for our antibody-based multiplex platform have been previously described [9].

### 2.3.2 Quantification of proteins using Western blot analysis

Western blot analysis was carried out using standard techniques. The concentrations of the antibodies used are documented in Supplementary Table 2.

### 2.4 Cell proliferation assays

#### 2.4.1 Sulforhodamine B (SRB) and CellTiter-Blue (CTB) assays

Cells were seeded in 96-well plates for 24 h before being treated with serial dilutions of the desired inhibitor, or vehicle control. The range of cetuximab concentrations used for the cell lines was 0.008 to 1000 µg/ml. After 72 h, cells were fixed using 10% trichloracetic acid, washed with double distilled water, air-dried and stained using 0.4% SRB in 1% acetic acid for 20 min. SRB was solubilized using 10 mM Tris–HCl and the absorbance was read at 570 nm wavelength using a TiterTec Multiscan MCC/340 MKII plate reader. For non-adherent cell lines (SNUC1) a CellTiter-Blue assay was used. For this, cells were seeded and treated in same way as per SRB assay. After 72 h CellTiter-Blue reagent was added at a 1:10 dilution and incubated for 4 h. Next, fluorescence was quantified using an EnVison plate reader. GI50 values were calculated using GraphPad prism 8.0.

#### 2.4.2 Clonogenic assay

Cells were seeded into 12-well plates (2.5 × 10³ to 7.5 × 10³ /well) and 24 h later inhibitors were added to the cells and incubated for 14 days. A concentration of GDC0941 and AZD2014 of 100 nM was chosen based on preliminary experiments that confirmed downstream signalling inhibition at the chosen dose (e.g. pPRAS40 and pS6, see Supplementary Fig. 2). Next, cells were fixed with 4% formaldehyde/PBS for 20 min and stained with 0.5% crystal violet in 70% ethanol. Finally, plates were imaged using a GelCount (Oxford Optronix) system.

### 2.5 Statistical tests and interpretation of multiplex phospho-proteomics data

For each of the cell lines used for both the 1 and 4 h experiments, three control samples and one cetuximab sample were used. All phospho-proteomic data were normalised to GAPDH. The mean and standard deviation (SD) of the median fluorescent intensity (MFI) were calculated for the three controls. For each cell line, when the cetuximab treated value was > 2 SDs above the mean of the controls it was classified as ‘increased’ and when it was < 2 SDs below the mean of the controls it was classified as ‘decreased’. If the cetuximab treated value was within 2 SDs of the mean of the controls it was considered unchanged. We chose to use this method for our cell line screens, as we did not validate
the linearity of the absolute changes in phospho-proteins in our assays.

For the ex vivo patient sample experiments, due to limited cell numbers, both 1 and 4 h experiments were not possible. Therefore, we were only able to carry out 1 h exposure experiments. The ex vivo experiments were otherwise conducted in the same way as the cell line experiments. For each patient sample we included 3 untreated controls and one drug exposed sample. Then the ratios of the cetuximab treated to untreated controls were calculated using the following equation: ratio of change = MFI of cetuximab treated sample / mean MFI of three controls. In red, phospho-proteins whose expression is increased, defined as having a cetuximab treated value that was at least 2 S.D. above the mean of three untreated controls. In blue, phospho-proteins whose expression is decreased, defined as a cetuximab treated value of at least 2 S.D. below the mean of controls. In white, phospho-proteins that were unchanged compared to controls. The black arrows indicate the phospho-protein changes that are statistically significant after Benjamini–Hochberg (BH) correction ($p < 0.05$). Phosphorylated RPS6 expression was statistically more likely to be downregulated in cetuximab sensitive cell lines following 1 h cetuximab exposure. At 4 h exposure phospho-PRAS40 was more likely to be decreased in sensitive cell lines, whereas pPDGFRα and pCHK2 were more likely to be increased in cetuximab resistant cell lines.

### Figure 2

Changes in phospho-protein levels in cetuximab sensitive and resistant KRAS/NRAS/BRAFV600 WT CRC cell lines. Phosphoprotein changes following 1 (a) and 4 (b) h cetuximab exposure. In red phospho-proteins whose expression is increased, defined as having a cetuximab treated value that was at least 2 S.D. above the mean of three untreated controls. In blue, phospho-proteins whose expression is decreased, defined as a cetuximab treated value of at least 2 S.D. below the mean of controls. In white, phospho-proteins that were unchanged compared to controls. The black arrows indicate the phospho-protein changes that are statistically significant after Benjamini–Hochberg (BH) correction ($p < 0.05$). Phosphorylated RPS6 expression was statistically more likely to be downregulated in cetuximab sensitive cell lines following 1 h cetuximab exposure. At 4 h exposure phospho-PRAS40 was more likely to be decreased in sensitive cell lines, whereas pPDGFRα and pCHK2 were more likely to be increased in cetuximab resistant cell lines.

### 3 Results and discussion

Six cell lines (COLO320, SW48, SNUC1, HCA24, C10 and CACO2) were classified to be cetuximab resistant, as a GI$_{50}$ was not reached at any concentration, with the highest cetuximab concentration used being 1000 µg/ml. Another six cell lines (C70, OXCO2, DiFi, NCIH508, LIM1215 and HCA46) were classified as cetuximab sensitive; mean ($\pm$ S.E) cetuximab GI$_{50}$ 0.063 ($\pm$ 0.025) µg/ml. The cetuximab GI$_{50}$
A phosphoproteomic study of cetuximab resistance in KRAS/NRAS/BRAF V600 wildtype colorectal cancer (CRC) cell lines was performed to understand the mechanism of cetuximab resistance. A number of phospho-proteins was found to be differentially regulated in the cetuximab sensitive versus resistant cell lines following 1 and 4 h exposure to 306 µg/ml cetuximab, a concentration that was clinically achievable in C max concentration trials. Binary logistic regression and adjustments for multiple testing were applied to test for changes that were statistically significant (Fig. 1). A decrease in phospho-RPS6 levels was statistically more likely to occur in cetuximab sensitive cell lines following 1 h of cetuximab exposure (p = 0.04). After a 4 h exposure another closely related PI3K pathway phospho-protein, phospho-PRAS40, was more likely to be decreased in cetuximab sensitive compared to resistant cell lines (p = 0.04). Conversely, phospho-PDGFRα and phospho-CHK2 were found more likely to be increased in cetuximab resistant versus sensitive cell lines (p = 0.04 and p = 0.03, respectively). Interestingly, phospho-EGFR levels were reduced in both cetuximab sensitive and resistant cell lines at a comparable level, which suggests that cetuximab continued to inhibit its intended target and that differences in sensitivity were possibly related to downstream signalling.

In 4 patient-derived samples, cells were immunomagnetically isolated from serous effusions. The clinical history of all 4 patients was suggestive of resistance to cetuximab (Fig. 3a). Changes in all phospho-proteins following cetuximab exposure are presented as ratios of changes compared to the untreated controls (Fig. 3b). The 1 h patient sample and cetuximab resistant cell line results were largely concordant. For example, a lack of reduction in phospho-RPS6 in 3 of 4 patient samples following exposure to cetuximab was seen in both resistant cell lines and patient samples. Interestingly, an increase in phospho-AKT Ser473 levels was seen in 3 out of 4 patient samples following exposure to cetuximab, suggesting continued activation of the PI3K pathway.

In view of the findings in the cell lines which showed that phospho-RPS6 and phospho-PRAS40 levels were not reduced in response to cetuximab in the cetuximab resistant cell lines, when compared to the cetuximab sensitive cell lines, and the finding that phospho-AKT Ser473 was increased in 3 out of 4 CRC samples exposed to cetuximab ex vivo for 1 h, we decided to explore the effects of the addition of PI3K pathway inhibitors to cetuximab ex vivo. We observed that in 3/4 of our samples isolated from CRC patients with established cetuximab resistance showed increases in phospho-AKT levels when exposed ex vivo to cetuximab. We subsequently investigated the

The role of KRAS/NRAS mutations in cetuximab resistance is well-documented and they lead to continued signalling downstream of EGFR through the PI3K and MEK-ERK pathways. The response rates of cetuximab monotherapy in patients with chemotherapy resistant KRAS WT CRC is, however, only ~20% and therefore there remains an unmet need to improve their clinical outcome. We aimed to investigate the role of signalling adaptations by determining sensitivity to cetuximab in KRAS WT CRCs. This is the first report of an antibody-based phospho-proteomic screen of dynamic changes in signal transduction ex vivo in cancer cells derived from patients with cetuximab resistant tumours. However, we had access to only a limited number of samples. Indeed, current literature suggests that patients with KRAS mutant tumours are more likely to develop serous effusions, a phenomenon that we also observed before. This may have contributed to the relatively small number of samples of cells isolated from patients with KRAS/NRAS/BRAF V600 WT CRC tumours that we were able to include.

Previous studies that investigated adaptations in signalling pathways as a mechanism of resistance to cetuximab suggested activation of receptor tyrosine kinases such as HER3 and non-receptor kinases such as SRC. While in our screen phospho-HER3 and SRC levels were increased in a number of cetuximab resistant cell lines following 1 h and 4 h of cetuximab exposure, respectively, the results were not statistically significant upon correction for multiple testing. This may at least in part be due to differences between the cell lines and patient samples used. For example, in studies identifying SRC as a regulator of cetuximab sensitivity/resistance, KRAS mutant cell lines such as HCT116 and HT29 were used. Instead in our data set, when corrected for multiple testing, only 4 changes were significant of which two (phospho-RPS6 and phospho-PRAS40) were related to the PI3K pathway. We speculate that in different cetuximab resistant cells, different receptor and non-receptor tyrosine kinases may be activated, with co-activation of several receptors being likely. This may subsequently result in the observed PI3K pathway upregulation. Indeed 3/4 of our samples isolated from CRC patients with established cetuximab resistance showed increases in phospho-AKT levels when exposed ex vivo to cetuximab. We subsequently investigated the

In the combination experiments in excess of that seen with single agents was modest and was not consistent across all cell lines. We also investigated the effects of the combination of 10 µg/ml cetuximab and the two PI3K pathway inhibitors using 14 day clonogenic assays. We observed a statistically significant higher inhibition of colony forming capacity following the addition of PI3K pathway inhibitors to cetuximab, when compared to single agent exposure, in all three cetuximab resistant cell lines (Fig. 4).

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effects of the combination of PI3K pathway inhibitors with cetuximab in three cetuximab resistant cell lines and observed additional growth inhibition when compared to single agent treatment activity.

Activation of the PI3K pathway in KRAS mutated CRC has been reported before [9, 19], but little is known about this in the KRAS/NRAS/BRAFV600 WT setting [20]. Factors that have been thought to be important in a KRAS/BRAFV600 WT setting include CCR7 expression [21] and mutations in genes regulating the PI3K pathway [22]. In contrast, another study has suggested that signalling through the PI3K pathway may confer a better response to anti-EGFR pathway treatment in this setting [23].

Other groups have previously suggested that PI3K pathway inhibitors such as PI3K, AKT and m-TOR inhibitors may be effective when combined with cetuximab, which is in agreement with our current data [24, 25]. Clinical trials have explored the combination of P13K pathway inhibitors (e.g. PX-886) with cetuximab in the KRAS WT setting without any improvement in clinical efficacy, but with increased toxicity in the combination over cetuximab alone [26]. On the other hand, a clinical trial that investigated addition of the m-TOR inhibitor everolimus to cetuximab and irinotecan

**Fig. 3** Clinical features and phospho-proteomic changes in cancer cells isolated from ascites/pleural effusions. a: Clinicopathological characteristics of patients at the time of sample collection, including the lines of systemic therapy received. Mutation data are based on standard diagnostics carried out on primary tumour samples. b: Cells were immunomagnetically isolated from patients’ serous effusions and were treated ex vivo with 306 µg/ml cetuximab or left untreated (n=4) for 1 h. Mean ratios of change (cetuximab treated to untreated controls) for all phospho-proteins quantified in 4 KRAS/NRAS/BRAFV600 wild-type CRC patient samples are shown. A ratio >1 (in red) suggests increase in phospho-protein expression in the cetuximab treated cells compared to the mean of controls and a ratio <1 (blue) the reverse. A lack of reduction in phospho-RPS6 in 3 of 4 patient samples following exposure to cetuximab was observed (lower arrow). In addition, an increase in phospho-AKT Ser473 levels was observed in 3 of 4 cell patient samples (upper arrow) following exposure to cetuximab, suggesting continued activation of the PI3K pathway.
in 43 patients suggested that the combination was clinically active and that it should be further developed [27]. Combinations of PI3K pathway inhibitors and cetuximab in the *KRAS/NRAS/BRAF* V600 WT setting should be explored in vivo along with biomarkers (e.g. PTEN loss) that may further enrich clinical benefit. These efforts may lead to combinations which are clinically actionable.

Our phospho-proteomic screen, though not extensive, did uncover actionable signalling nodes from multiple different signal transduction pathways such as receptor tyrosine kinases (RAS-RAF-MEK, PI3K-AKT-m-TOR), angiogenesis receptors (JAK-STAT, WNT) and DNA damage repair pathways (supplementary Fig. 1). It is of interest to note that upon correction for multiple testing it was mainly the PI3K pathway that showed differences in signalling between cetuximab sensitive and resistant lines at early time points. This offers insights into differences in dependencies on signal transduction pathways between...
cetuximab resistant and sensitive KRAS/NRAS/ BRAFV600 WT CRC.

We also report dynamic signalling patterns in freshly isolated cancer cells from serous effusions in patients with KRAS/NRAS/BRAFV600 WT CRC who were resistant to cetuximab therapy. Though the number of samples was small, it was encouraging to see concordance between activation of the PI3K pathway between the cell lines and patient samples in the assays studying early changes in dynamic signal transduction.

We acknowledge some limitations of our work. All cell lines were cultured to 70% confluence before 1 or 4 h exposure to control or cetuximab. This may have led to differences in the actual cell numbers seeded between cell lines at the time of cetuximab dosing. This was compensated by seeding equal amounts of protein in our phospho-proteomic experiments for all cell lines and patient samples used. Moreover, all phospho-proteomic results were adjusted for loading using GAPDH. In our 25 cell line 1 and 4 h cetuximab exposure experiments and in our 1 h ex vivo patient sample experiments, one cetuximab exposed and three untreated control samples were used. This was compensated by our strict definition of a ‘hit’ in our phospho-proteomic analysis. A significantly altered phospho-protein expression was defined as a treated value of 2 standard deviations above or below the mean of the three controls. Moreover, when comparing cetuximab sensitive and resistant cells, our statistical analysis for significance included adjustment for multiple testing. Lastly, the patient sample genomic data were derived from analysis of the primary tumours and this was not confirmed in the malignant cells isolated from the serous effusions. We acknowledge that tumour heterogeneity and effects of previous treatments may have affected these genomic aberrations. Nevertheless, there is evidence to suggest that there is high genomic concordance between matched primary and metastatic CRC samples [28].

In conclusion, our phospho-proteomic study of acute rewiring of signal transduction, following exposure to clinically relevant concentrations of cetuximab, in a panel of KRAS/NRAS/BRAFV600 WT cell lines and ex vivo in samples derived from patients with clinical resistance to cetuximab, strongly suggests continued activation of the PI3K pathway as a mechanism of resistance to cetuximab. Combinations of PI3K pathway inhibitors and cetuximab in the KRAS/NRAS/ BRAFV600 WT setting should be explored in vivo, which may lead to combinations that are clinically actionable.

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Authors’ contributions Conceptualization: A.G., S.W. and U.B.; methodology: A.G., A.S., S.W. and U.B.; data collection: A.G., A.S. G.V. and L.P.; data analysis: A.G., A.S., L.P. and G.V.; writing–original draft preparation: A.G.; writing–review and editing: A.G., A.S., G.V., D.C., N.V., S.W. and U.B.; supervision: S.W. and U.B.

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Declarations

Conflict of interest A.G., A.S., G.V., L.P. and S.W. declare no conflicts of interest. D.C. has received grants from 4SC, Amgen, AstraZeneca, Bayer, Celgene, Clovis, Eli Lilly, Janssen, Medimmune, Merck, Merrimack and Sanofi outside the submitted work. N.V. received honoraria and/or travel accommodation from Merck Serono, Pfizer, Bayer, Eli-Lilly and Menarini Silicon Biosystems and U.B. has received honoraria from Astellas, Novartis, Karus Therapeutics, Phoenix Solutions, Eli Lilly, Astex, Vernalis, Janssen and Boehringer Ingelheim. His institutional financial interests include funding for Phase 1 investigator-initiated trials from Onyx Pharmaceuticals, BTG International, Carrick Therapeutics, Chugai, AstraZeneca and Verastem. He is an employee of the Institute of Cancer Research, London, which is involved in the development of PI3K, HSP90, HDAC, AKT, ROCK, RAF, CHK1 and HSF1 inhibitors.

Ethical approval The study was carried out in accordance with the declaration of Helsinki and approved by a human research ethics committee.

Informed consent All patients provided written informed consent prior to their participation.

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