



# Simulating and predicting cellular and in vivo responses of colon cancer to combined treatment with chemotherapy and IAP antagonist Birinapant/TL32711

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## Abstract

Apoptosis resistance contributes to treatment failure in colorectal cancer (CRC). New treatments that reinstate apoptosis competency have potential to improve patient outcome but require predictive biomarkers to target them to responsive patient populations. Inhibitor of apoptosis proteins (IAPs) suppress apoptosis, contributing to drug resistance; IAP antagonists such as TL32711 have therefore been developed. We developed a systems biology approach for predicting response of CRC cells to chemotherapy and TL32711 combinations in vitro and in vivo. CRC cells responded poorly to TL32711 monotherapy in vitro; however, co-treatment with 5-fluorouracil (5-FU) and oxaliplatin enhanced TL32711-induced apoptosis. Notably, cells from genetically identical populations responded highly heterogeneously, with caspases being activated both upstream and downstream of mitochondrial outer membrane permeabilisation (MOMP). These data, combined with quantities of key apoptosis regulators were sufficient to replicate in vitro cell death profiles by mathematical modelling. In vivo, apoptosis protein expression was significantly altered, and mathematical modelling for these conditions predicted higher apoptosis resistance that could nevertheless be overcome by combination of chemotherapy and TL32711. Subsequent experimental observations agreed with these predictions, and the observed effects on tumour growth inhibition correlated robustly with apoptosis competency. We therefore obtained insights into intracellular signal transduction kinetics and their population-based heterogeneities for chemotherapy/TL32711 combinations and provide proof-of-concept that mathematical modelling of apoptosis competency can simulate and predict responsiveness in vivo. Being able to predict response to IAP antagonist-based treatments on the background of cell-to-cell heterogeneities in the future might assist in improving treatment stratification approaches for these emerging apoptosis-targeting agents.

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## Introduction

Stage III and high-risk stage II colon cancer patients receive adjuvant 5-fluorouracil (5-FU)-based chemotherapy often combined with oxaliplatin. However, 5-FU/oxaliplatin treatment in stage III benefits only 15–20% of patients [1]. Moreover, 5-year overall survival (OS) rates

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are less than 6% for stage IV metastatic colorectal cancer (mCRC) patients treated primarily with 5-FU-based regimens. Current targeted treatments such as anti-EGFR therapies are approved in the metastatic setting only for a subset of patients (*RAS*, *BRAF* wild-type) and are ineffective in the adjuvant setting [2, 3]. Since pre-existing or acquired resistance to apoptosis significantly contributes to treatment failure in cancer [4], the evaluation of new treatment combinations which reinstate apoptosis competency has the potential to improve patient outcome.

Novel targeted drugs which neutralise apoptosis-inhibiting proteins have potential as enhancers of chemotherapy. The group of intracellular anti-apoptotic proteins is relatively small, with caspase-8/-10 inhibitor FLIP, anti-apoptotic Bcl-2 family members and inhibitor of apoptosis (IAP) proteins being the major players. The Bcl-2 antagonist venetoclax/ABT-199 has recently been approved for the treatment of patients with 17p deleted chronic lymphocytic leukaemia and is currently being tested in additional cancers [5]. From the group of IAP antagonists that have been evaluated, clinical studies have shown that TL32711/Birinapant (Tetralogics) and LCL161 (Novartis) can be combined safely with a range of chemotherapeutic agents, and both have entered phase 2 trials (<http://clinicaltrials.gov/>) [6]. TL32711 generated responses in combination with irinotecan in a subset of colorectal cancer patients who were refractory to irinotecan alone [7]. Such response heterogeneities indicate that stratification tools and response predictors will be required to preselect patients likely to respond to IAP antagonist-based combination treatments.

IAP antagonists were initially designed to replicate the function of second mitochondria-derived activator of caspases (SMAC) in binding to and blocking X-linked inhibitor of apoptosis protein (XIAP), the major antagonist of proteases essential for efficient apoptosis execution (caspases-9, -3 and -7) [8]. IAP antagonists also bind to and trigger the rapid degradation of cellular IAP (cIAP) 1 and 2 [9], both of which are critical regulators of ripoptosome formation and caspase-8-dependent apoptosis induction in response to intrinsic pro-apoptotic stress and activation of tumour necrosis factor receptor (TNFR) family [6, 8]. Correspondingly, IAPs have been implicated as mediators of drug resistance in various cancers, including colorectal cancer [10, 11].

In this study, we obtained a single cell understanding of signal transduction kinetics and heterogeneities for treatments based on combinations of 5-FU/oxaliplatin and TL32711, and applied a systems biology strategy towards

predicting the resulting cell death patterns in populations of CRC cells in vitro and in vivo.

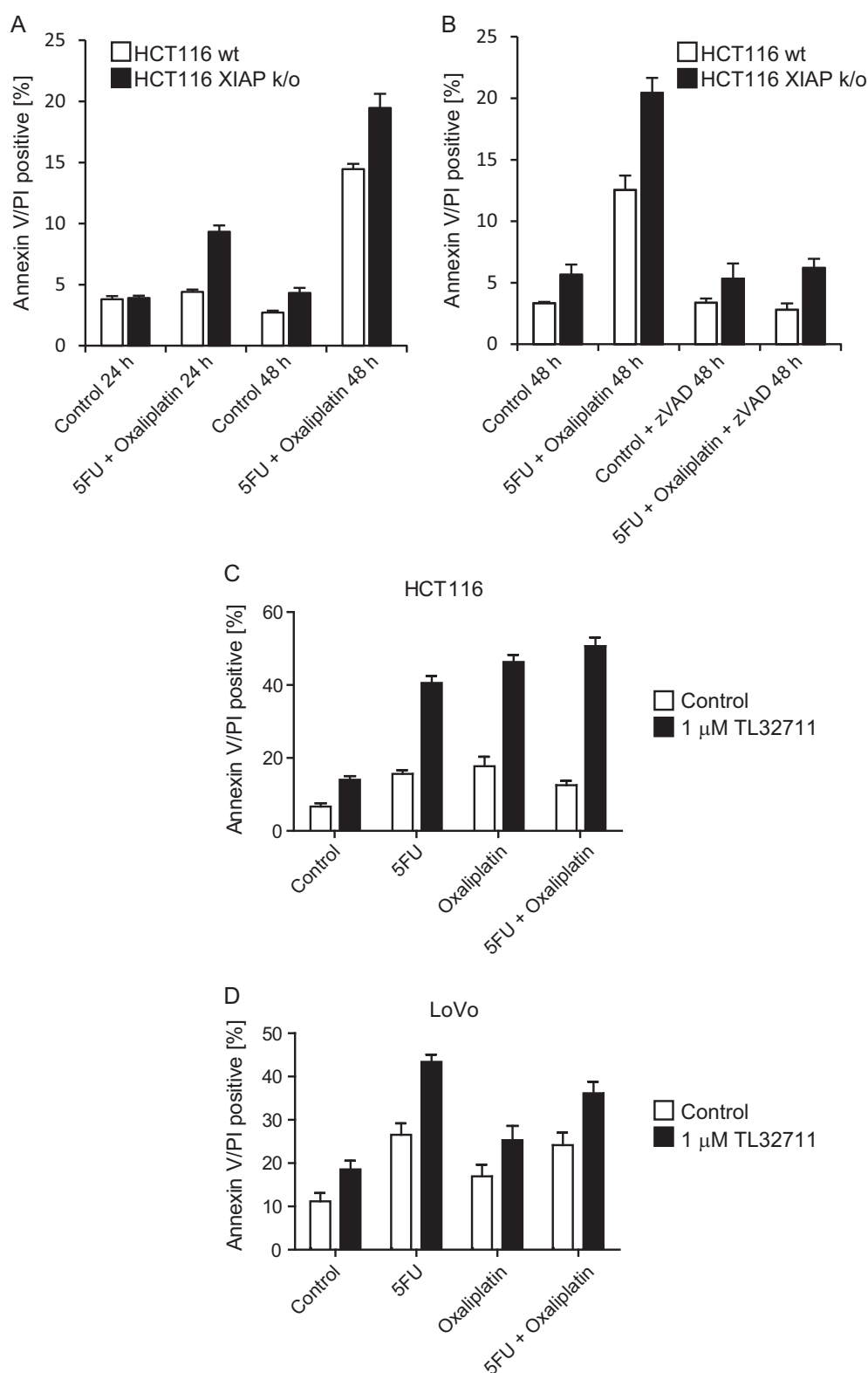
## Results

### IAP antagonist TL32711/Birinapant sensitises CRC cell lines to chemotherapy-induced cell death

XIAP is implicated as an important mediator of clinical drug resistance [12]. We assessed the role of XIAP in the apoptotic response of CRC cells to the therapeutic combination of 5-FU and oxaliplatin. Genetic loss of XIAP sensitised HCT116 cells to cell death induced by 5-FU/oxaliplatin after 48 h of treatment (Fig. 1a). The cell death induced by 5-FU/oxaliplatin was caspase-dependent (implying apoptosis) since the pan-caspase inhibitor zVAD-fmk abolished cell death in both parental and XIAP null cells (Fig. 1b). These results indicate that XIAP is an important mediator of resistance to 5-FU/oxaliplatin. We therefore co-treated HCT116 cells with 5-FU and oxaliplatin alone and in combination in the presence or absence of the IAP antagonist TL32711 and determined cell death. In agreement with results in XIAP null cells, cell death was significantly increased by addition of TL32711 (Fig. 1c). Similar results were obtained in the LoVo CRC model, with TL32711 enhancing apoptosis induced by 5-FU, oxaliplatin and the combination treatment (Fig. 1d). In both cell lines, TL32711 alone had a relatively modest effect on cell death.

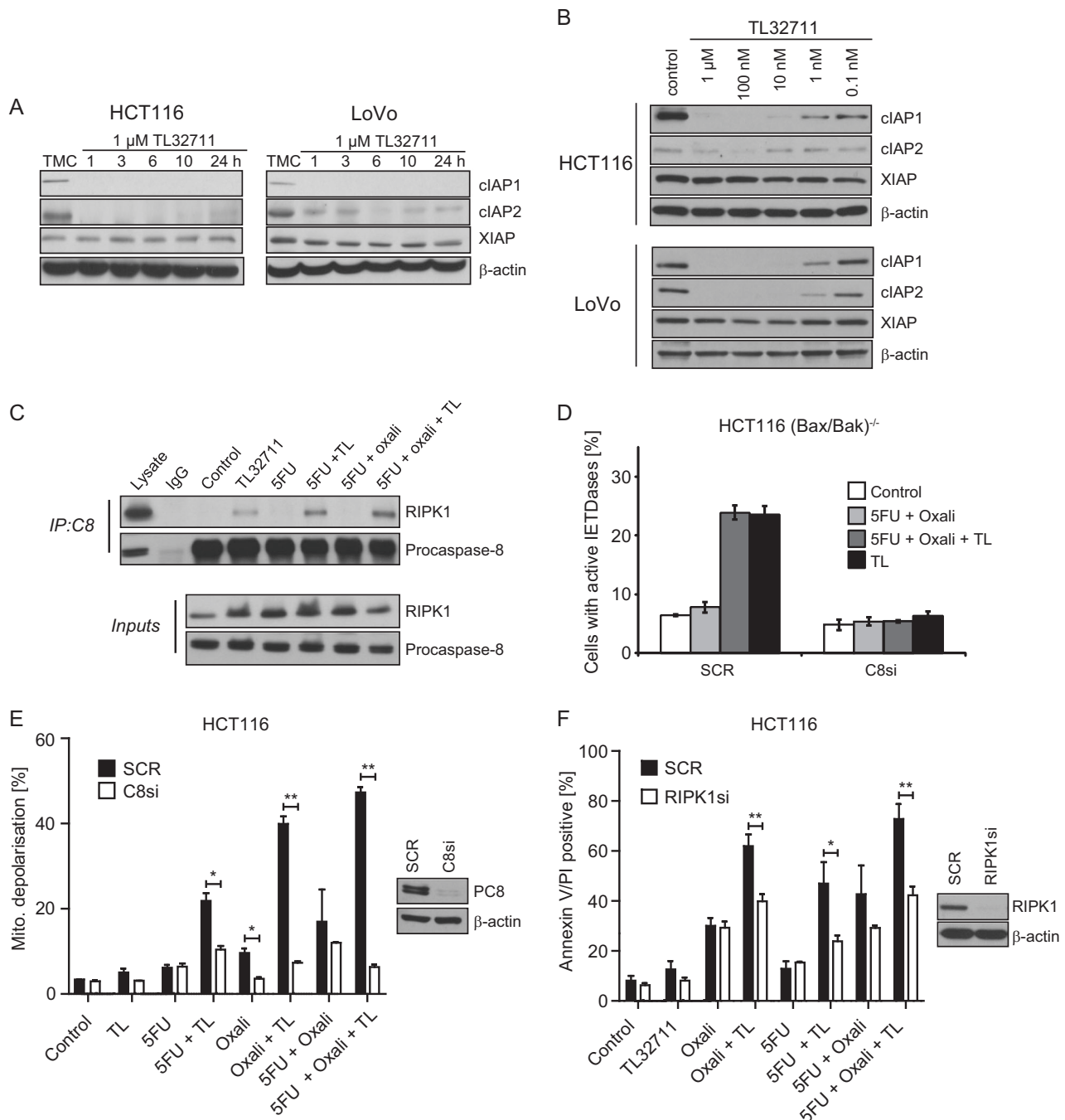
### IAP antagonist TL32711 promotes cIAP1 and cIAP2 depletion, formation of complex II and caspase-8/RIPK1-dependent cell death

We next further characterised the consequences of and the molecular signalling events initiated by TL32711 treatment. In both HCT116 and LoVo cells, TL32711 caused the rapid and persistent downregulation of cIAP1 (Fig. 2a). In many cell lines, cIAP2 expression recovers after initial rapid degradation; this is because cIAP1 acts as an E3 ligase for cIAP2, and degradation of cIAP1/2 leads to activation of the non-canonical NFκB pathway resulting in increased expression of NFκB-driven genes, including cIAP2 [13, 14]. It was therefore notable that cIAP2 expression was also persistently downregulated in both cell line models in response to TL32711. It is known that TL32711 also inhibits XIAP ( $K_D = 45$  nM) [15], the most potent caspase-inhibiting IAP that directly targets caspases-3, -7 and -9, XIAP expression was relatively unaffected by TL32711 in both cell lines (Fig. 2a); this is



**Fig. 1** XIAP depletion and IAP antagonist TL32711 sensitise CRC cell lines to chemotherapy-induced cell death. **a** AnnexinV/PI staining of HCT116 and HCT116 xiap<sup>-0</sup> cells treated with 5FU (10  $\mu$ M) and oxaliplatin (2  $\mu$ M) for up to 48 h. **b** AnnexinV/PI staining of HCT116 and HCT116 xiap<sup>-0</sup> cells treated with oxaliplatin and 5-FU for up to

48 h in presence or absence of 20  $\mu$ M pan-caspase inhibitor z-VAD-fmk. **c, d** Annexin V/PI staining of HCT116 and LoVo cells 48 h following single or combination treatment with 10  $\mu$ M 5-FU, 2  $\mu$ M oxaliplatin and 1  $\mu$ M TL32711. All data are shown as mean  $\pm$  s.e.m. from  $n = 3$  independent experiments



**Fig. 2** IAP antagonist TL32711 promotes cIAP1,2 depletion, formation of complex II and caspase-8/RIPK1-dependent cell death. **a** Western blot analysis of cIAP1, cIAP2 and XIAP expression in HCT116 and LoVo cells following treatment with 1  $\mu$ M TL32711.  $\beta$ -actin served as loading control. **b** Western blot analysis of cIAP1, cIAP2 and XIAP 24 h following treatment with varying concentrations of TL32711 in HCT116 and LoVo cells.  $\beta$ -actin served as loading control. **c** Western blot analysis of RIPK1 and procaspase-8 following caspase-8 immunoprecipitation 48 h following treatment with 10  $\mu$ M 5-FU, 2  $\mu$ M oxaliplatin and 3 h after addition of 1  $\mu$ M TL32711 in the presence of 10  $\mu$ M z-VAD-fmk in HCT116 cells. Input controls demonstrate presence of RIPK1 and procaspase-8 in all lysates. **d** Caspase-8-like (IETDase) activity was measured in HCT116 (Bax/Bak)<sup>-/-</sup> cells expressing a CFP-IETD-YFP FRET probe and that were

transfected with scrambled control (SCR) siRNA or siRNA targeting procaspase-8 (C8si). Data are shown as mean  $\pm$  s.d. from triplicates. Experiment was repeated twice with similar results. **e** Mitochondrial depolarisation as determined by loss of TMRE uptake in HCT116 cells transfected with scrambled control (SCR) siRNA or siRNA targeting procaspase-8 (C8si). Cells were (co-)treated with 1  $\mu$ M TL32711 (TL), 10  $\mu$ M 5-FU and 2  $\mu$ M oxaliplatin (oxali) for 48 h. Western blot insert demonstrates efficient depletion of procaspase-8 (PC8) expression. **f** Annexin V/PI staining of HCT116 transfected with scrambled control (SCR) siRNA or siRNA targeting RIPK1 and (co-)treated with 1  $\mu$ M TL32711 (TL), 10  $\mu$ M 5-FU and 2  $\mu$ M oxaliplatin (oxali) for 48 h. Western blot insert demonstrates efficient depletion of RIPK1 expression. \* $p$  < 0.05; \*\* $p$  < 0.01

consistent with the predominant effect of IAP antagonists on XIAP being disruption of its protein–protein interactions with caspases. In further dose–effect studies, we found that nanomolar concentrations of TL32711 were sufficient to cause the degradation of cIAP1 and cIAP2 in both cell lines (Fig. 2b).

The loss of cIAPs is known to promote the interaction of procaspase-8 with RIPK1, triggering the formation of complex II or the ripoptosome, a cytosolic caspase-8 activation platform that can trigger apoptosis. Genotoxic stress may also promote ripoptosome formation [16]. Assessment of ripoptosome formation indicated that treatment with TL32711 in combination with 5-FU increased RIPK1/procaspase-8 complex formation compared to treatment with TL32711 alone, and this was further enhanced when TL32711 was combined with 5-FU/oxaliplatin (Fig. 2c), suggesting that standard-of-care chemotherapy promotes ripoptosome formation in CRC cells in the context of IAP antagonist treatment (Fig. 2c). Importantly, this interaction was not observed when cells were exposed only to the chemotherapeutics (Fig. 2c), indicating that TL32711 initiates an additional pro-apoptotic signalling cascade in parallel to other genotoxic responses in these cells.

Next, we investigated whether the formation of procaspase-8/RIPK1 complexes also results in the activation of caspase-8. To determine initiator caspase-8 activity inside living HCT116 cells, we measured the cleavage of an exogenously expressed CFP-YFP fusion protein containing the preferred caspase-8 cleavage motif IETD by FRET flow cytometry [17, 18]. To avoid contributions of downstream effector caspases-3 and -7, which both also cleave after IETD motifs, we conducted these experiments in HCT116 cells deficient in Bax and Bak [19]. As shown in Fig. 2d, only treatments including TL32711 resulted in IETD probe cleavage in HCT116 cells. Furthermore, IETDase activity was completely eliminated when cells were transfected with an siRNA targeting procaspase-8 (Fig. 2d). We next determined whether caspase-8 activation would be required to trigger mitochondrial outer membrane permeabilisation (MOMP). Indeed, depletion of procaspase-8 expression abolished mitochondrial depolarisation in parental HCT116 cells, specifically in response to TL32711/chemotherapy combination (Fig. 2e). To complement these analyses, we also depleted RIPK1 expression by siRNA and determined cell death induction; notably, loss of RIPK1 expression also significantly reduced cell death in TL32711 combination treatments (Fig. 2f).

These findings demonstrate that the presence of TL32711 promotes the formation of complexes, presumably ripoptosomes, containing caspase-8 and RIPK1, resulting in upstream caspase-8 activation in a significant proportion of cells. In agreement with this, cell death co-

depends on the presence of both procaspase-8 and RIPK1.

### Single-cell real-time imaging identifies two distinct caspase activation patterns leading to apoptosis execution in TL32711-based combination treatments

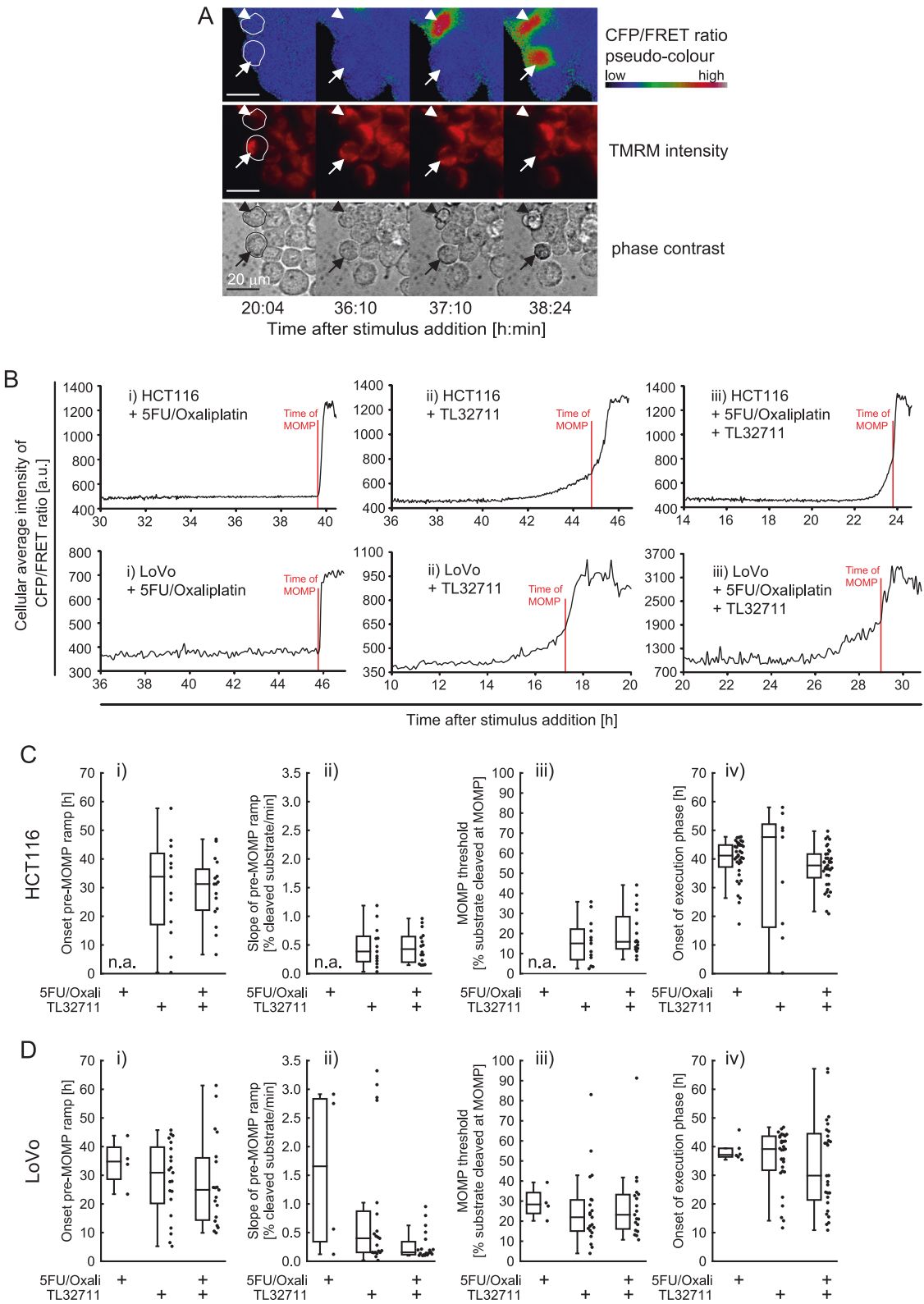
Next, we analysed the kinetics of intracellular apoptosis signal transduction in HCT116 and LoVo cells that responded to 5-FU/oxaliplatin and TL32711, alone or in combination. We expressed another CFP-YFP FRET probe, containing a DEVD linker motif preferentially cleaved by caspases-3/-7, but also cleavable by caspase-8 in HCT116 and LoVo cells [20]. Readings obtained from this probe (cleavage amounts and kinetics) are also suitable to validate predictions made by deterministic mathematical modelling of apoptosis signalling as to whether apoptosis can be executed efficiently in human cancer cells [21, 22]. Data obtained from these time-lapse imaging studies, in combination with the results from the previous experimental characterisations, provided a basis for the subsequent development of an extended mathematical framework for simulating the responsiveness of CRC cell populations to treatment combinations that include the IAP antagonist TL32711.

Cells stably expressing the FRET probe were treated and monitored over time by fluorescence microscopy, followed by ratiometric analysis of probe cleavage. As expected, individual cells responded with probe cleavage at different times after treatment (Fig. 3a). In parallel, cells were loaded with tetramethylrhodamine methyl ester (TMRM) to obtain data on mitochondrial transmembrane potentials, which depolarise upon MOMP and release of cytochrome c and can serve as a surrogate marker for timing the beginning of the apoptosis execution phase [21, 23]. For individual cells, fluorescence signals were extracted and plotted to obtain quantitative data on probe cleavage kinetics.

In our measurements, we could observe two prominent response patterns of FRET probe cleavage in single cells (Fig. 3b). In HCT116 cells, treatment with the chemotherapy combination of 5-FU/oxaliplatin resulted in classical post-MOMP patterns of FRET probe cleavage during apoptosis execution (Fig. 3bi). Only a few cells responded to TL32711, and these presented with an initial phase of slow, modest probe cleavage (Fig. 3bii). This “ramp” of substrate cleavage was followed by a transition towards rapid probe cleavage once the execution phase was triggered (Fig. 3bii). These patterns were reminiscent of cleavage kinetics previously described by us and others for caspase-8-dependent apoptosis initiation [17, 24]. Upon combination of chemotherapeutics with TL32711, these dual-phase cleavage patterns were also observed (Fig. 3biii)

and became more frequent. Nevertheless, a fraction of cells also responded with patterns comparable to chemotherapy-only treatments. Control cells which remained untreated did not display probe cleavage or significant changes in the

FRET signal, excluding the possibility that phototoxicity or probe bleaching contributed to the signals recorded (not shown). Similar findings were also made in LoVo cells (Fig. 3bi–iii)





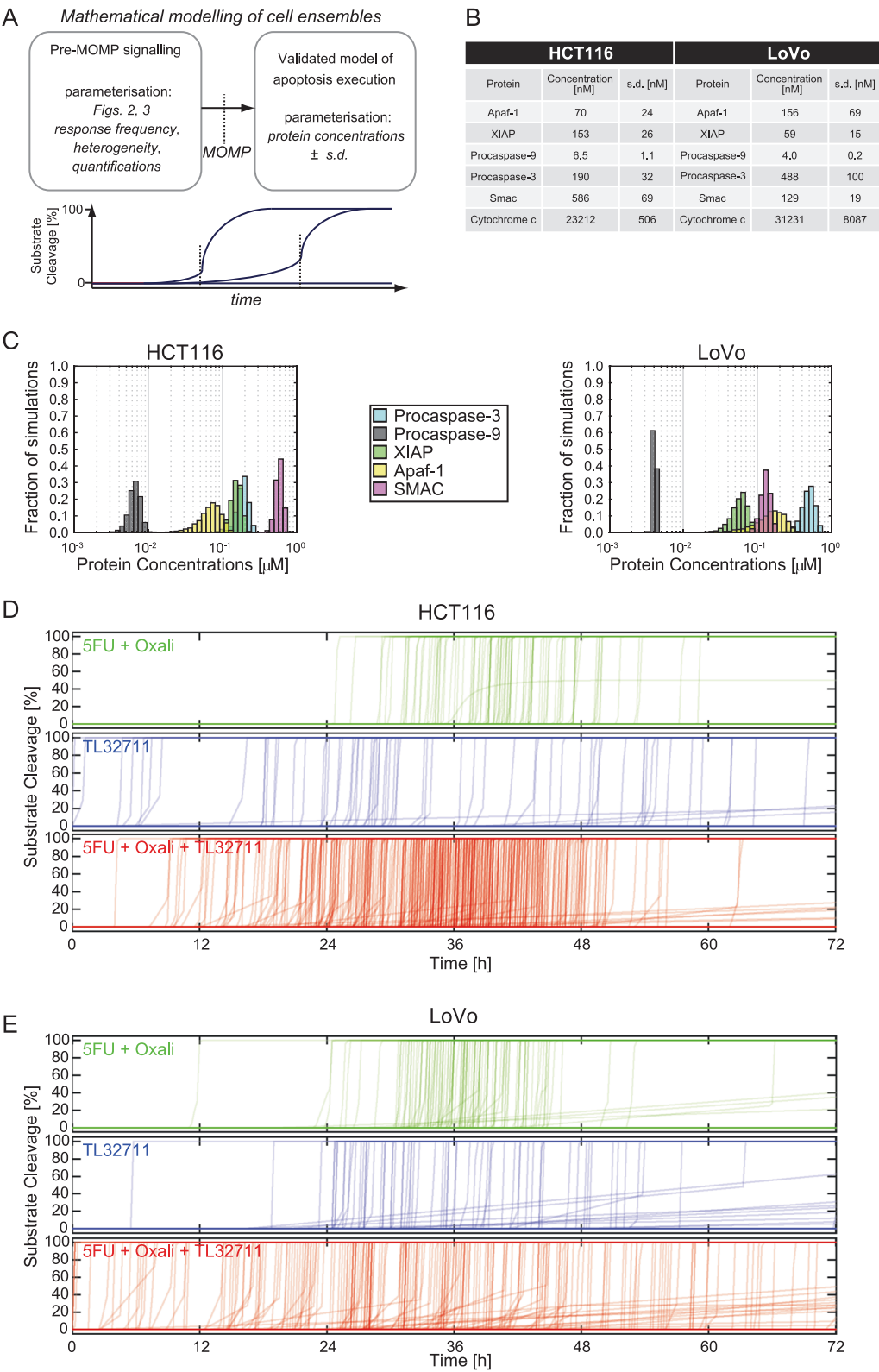
◀ **Fig. 3** Single-cell real-time imaging identifies two distinct caspase activation patterns leading to apoptosis execution in TL32711-based combination treatments. **a** Pseudo-coloured CFP/YFP emission ratio images of a group of HCT-116 cells expressing a DEVDase FRET probe treated with 5-FU/oxaliplatin (10  $\mu$ M/ 2  $\mu$ M) in combination with TL32711 (1  $\mu$ M). DEVDase activity resulted in FRET probe cleavage and displayed as an increase in the CFP/YFP emission ratio. Loss of tetramethylrhodamine methyl ester (TMRM) fluorescence indicates mitochondrial depolarisation as a consequence of MOMP. Time stamps indicate time after stimulus addition. **b** Exemplary DEVDase response profiles of HCT116 cells (upper panels) treated with 5-FU/oxaliplatin, TL32711 or the combination of both. Cells responding to 5-FU/oxaliplatin predominantly showed late DEVDase activity, subsequent to the onset of mitochondrial depolarisation and corresponding to the canonical activation of the intrinsic apoptosis pathway (i). Cells responding to TL32711 single treatment exclusively displayed response patterns resembling the one shown in (ii), with mild DEVDase activity converted into rapid substrate cleavage following onset of mitochondrial depolarisation. Cells treated with the combination of 5-FU/oxaliplatin and TL32711 frequently presented substrate cleavage ramps prior to the onset of mitochondrial depolarisation (iii), as well as response patterns without pre-MOMP caspase activity, resembling those shown in (i). Similar response characteristics were observed in LoVo cells (i–iii). Quantification of response types and kinetics for all **c** HCT116 and **d** LoVo cells responding to the treatments, the response characteristics were analysed and quantified. Parameters evaluated included the onset time of pre-MOMP caspase activity (time from drug addition to initiator caspase activation), the slope of pre-MOMP substrate cleavage as a measure of caspase activity, the mitochondrial MOMP threshold (amount of substrate cleaved at the time MOMP-induced mitochondrial depolarisation begins), as well as the onset time of the execution phase. Data are shown median  $\pm$  quartiles

An overall quantification of all responses is shown in Fig. 3c, d. We determined the onset time of probe cleavage ramps as well as the slope of these ramps, serving as an indicator for pre-MOMP caspase-8 activity. In addition, we determined the mitochondrial MOMP threshold (amount of substrate cleaved at MOMP) as well as the onset time of the post-MOMP execution phase (Fig. 3c, d). Overall, this demonstrated that moderate probe cleavage (“pre-MOMP ramp”), most likely due to caspase-8 activation (see Fig. 2), in HCT116 can primarily be observed in the presence of TL32711 (Fig. 3ci). In the LoVo model, cells with pre-MOMP substrate cleavage could also be found in response to 5-FU/oxaliplatin, and the fraction of these cells increased when TL32711 was combined with 5-FU/oxaliplatin (Fig. 3Di). We therefore conclude that response patterns within CRC cell populations are heterogeneous, with cells either displaying rapid FRET probe cleavage as expected for apoptosis execution, or initial moderate pre-MOMP caspase-8 activity, which subsequently is converted into rapid apoptosis execution.

## Mathematical simulations of apoptosis signalling can recapitulate death profiles and apoptosis sensitisation by IAP antagonist TL32711

Next, we established a mathematical modelling framework that can replicate the apoptosis heterogeneity observed in HCT116 and LoVo cell populations. To this end, we extended a previous deterministic ODE-based model of the apoptosis execution phase that captures the interplay of the apoptosome (cytochrome c-induced APAF-1 oligomerisation and caspase-9 activation), SMAC, XIAP and caspase-3 [21]. The terminal outputs of this model are simulated cleavage kinetics for DEVD caspase substrates, allowing the comparison of model outputs to time-lapse imaging data. Furthermore, we previously showed that >25% substrate cleavage during apoptosis execution is highly indicative of irreversible cell death induction [21]. For the model extension here, we included a function which replicates pre-MOMP caspase-8 activity and also implemented a threshold mechanism by which prolonged caspase-8 activity triggers apoptosis execution (see Fig. 4a and methods). Importantly, we developed this model so that it can capture the cell-to-cell heterogeneity observed experimentally in HCT116 and LoVo cell populations. This was achieved by parameterising *in silico* ensembles of 1000 cells for each treatment condition, where for each individual cell the respective initial model parameters were sampled from the distributions of experimental input data. To parameterise the reactant amounts, we determined the nM protein expression of key apoptosis regulators in HCT116 and LoVo cells by quantitative Western blotting (12-bit dynamic range) (Fig. 4b). For the model, these quantities were converted to normal distributions around the measured mean (Fig. 4c). Similarly, pre-MOMP signalling was parameterised with the experimentally determined heterogeneity data, including the frequency of upstream caspase-8 activation, onset times, activities and MOMP thresholds (see results in Figs 2, 3).

In the subsequent simulations for HCT116 cell populations, we noted that chemotherapy or TL32711 on their own induced apoptosis execution in rather few cells of the simulated ensembles (Fig. 4d). Calculating substrate cleavage and apoptosis execution for the combination of chemotherapy and TL32711 instead showed, as expected, that significantly more cells responded (Fig. 4d). Similar simulation results were obtained for LoVo cells, with the combination of chemotherapy and TL32711 again resulting in significantly enhanced apoptosis execution (Fig. 4e). These results therefore demonstrate that mathematical modelling can recapitulate apoptosis responses, the sensitising effects of TL32711 and cell-to-cell response heterogeneities observed experimentally in populations of CRC cells.





◀ **Fig. 4** Mathematical simulations of apoptosis signalling can recapitulate death profiles and apoptosis sensitisation by IAP antagonist TL32711. **a** Overview of the architecture of the developed model. Data from experimental analyses of signalling kinetics upstream of MOMP were used to construct mathematical functions replicating early signalling characteristics. These then feed into and trigger the execution phase of apoptosis. The execution phase was simulated using a validated model based on coupled ordinary differential equations. The primary model output reflecting cellular apoptosis competency is the calculated amount of cellular substrates cleaved by caspases. A substrate cleavage above 25% is considered to be sufficient for successful apoptosis execution. The diagram insert shows exemplary cell behaviours, such as non-responsive cells and cells responding at different times and with different SC thresholds for MOMP induction. See methods section for details. **b** Mean expression of key players of apoptosis execution as quantified in HCT116 and LoVo cells from  $n = 3$  experiments using quantitative immunoblotting. **c** Cellular protein expression converted into normal distributions. Random sampling from these distributions served to parameterise the apoptosis execution module of the mathematical model. The behaviour of ensembles of 1000 cells per treatment condition was mathematically modelled for HCT116 cells (**d**) or LoVo cells (**e**). Cell ensembles were parameterised as described in the methods and results sections. Each line represents the behaviour of one cell in the modelled ensemble. Near-vertical lines indicate phases of rapid substrate cleavage as observed during apoptosis execution. Ramps represent phases of pre-MOMP substrate cleavage. Modelling results replicate experimental findings: Cells heterogeneously respond with apoptosis induction and execution, with combination treatment of chemotherapy and TL32711 causing higher amounts of cell death

## Mathematical modelling predicts increased apoptosis resistance of xenografted HCT116 and

### LoVo cell tumours, which can be overcome by chemotherapy/TL32711 co-treatment

Next, we assessed whether our mathematical modelling approach would allow us to calculate in vivo apoptosis sensitivity and effects on tumour growth in response to 5-FU/oxaliplatin and TL32711. We established xenograft tumour growth models for HCT116 and LoVo cells. Since protein expression amounts in HCT116 and LoVo cells may differ between in vitro and in vivo growth conditions, we first collected tumours grown in untreated control mice for protein quantification. Interestingly, we noted significant differences in apoptosis protein expression amounts between the two growth conditions in both HCT116 and LoVo cells (Fig. 5a–c). In HCT116 cells, notable decreases in SMAC (1.7-fold), APAF-1 (4.7-fold) and Procaspase-9 (2.1-fold) expression were detected, whereas XIAP expression increased (3.4-fold) (Fig. 5a–c). We made similar findings in tumours grown from LoVo cells, where again decreases in APAF-1 (3.8-fold) and procaspase-9 (2.7-fold) expression and increases in XIAP (2.1-fold) expression were observed, while procaspase-3 expression also decreased (1.7-fold) in these tumours (Fig. 5a–c). We

therefore used the in vivo protein expression data to re-parameterise our model and to calculate whether apoptosis responsiveness of HCT116 and LoVo cell ensembles would be affected. Results from these simulations suggested that for in vivo conditions, poor responsiveness to both chemotherapy and TL32711 would be expected, indicating that the changes in protein expression observed in xenograft tumours impair apoptosis execution in a significant fraction of the populations (Fig. 5d, e, compare to Fig. 4d, e). The expected in vivo heterogeneity in execution efficacy is more explicitly shown in Supplemental Fig. 1 (Fig. S1). Despite the strong trend towards higher apoptosis resistance, the simulation results also show that the combination of chemotherapy plus TL32711 would notably enhance cell death responses in HCT116 and LoVo xenografts (Fig. 5d, e; Fig. S1).

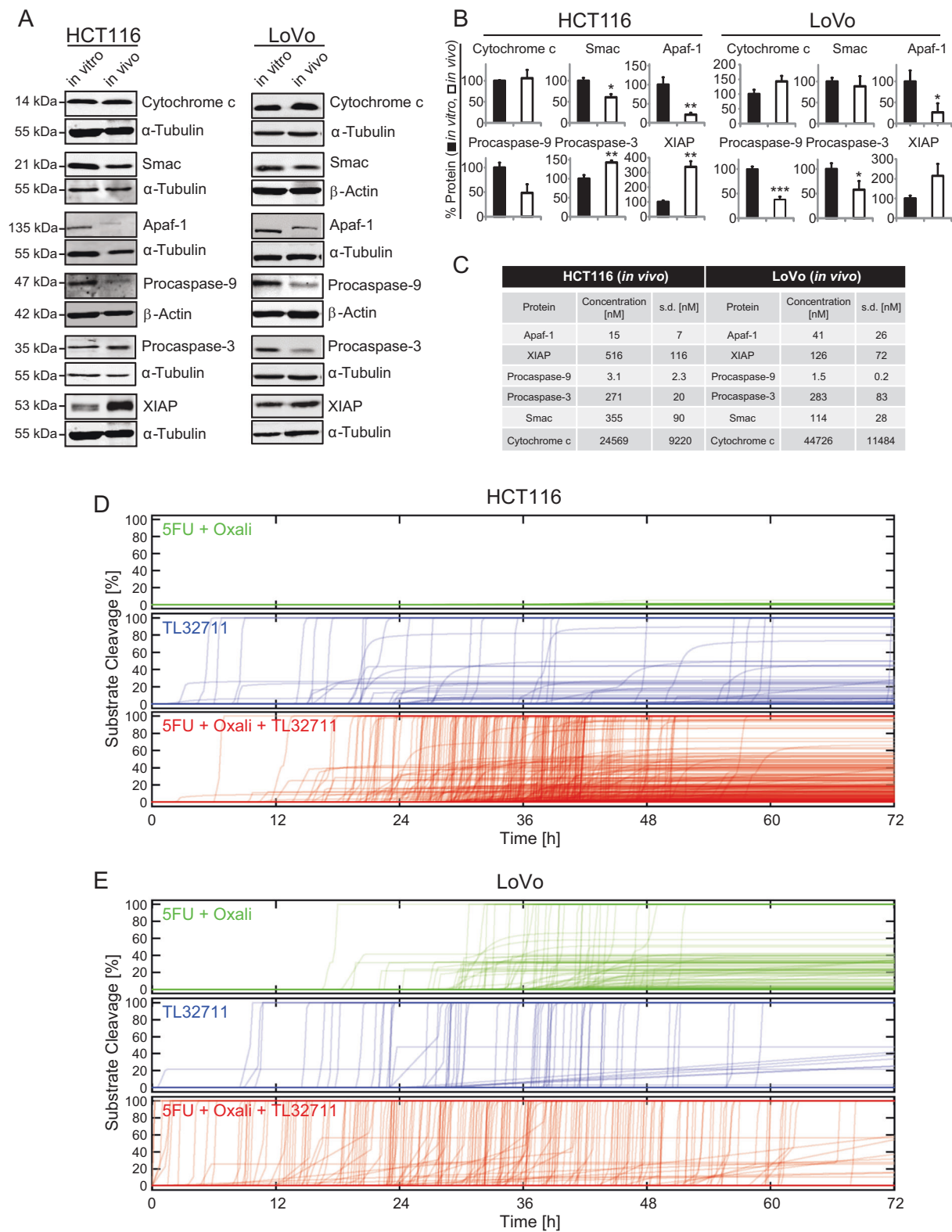
## Mathematical modelling captures drug effects observed in vivo and predicts treatment responses

Cell death events accumulated from the cell ensemble simulations (Fig. 5d, e) indicated that under in vivo conditions, the response of HCT116 and LoVo xenografts to chemotherapy and TL32711 combinations is considerably higher than for single treatment (Fig. 6a, b). To validate the model predictions, we next treated mice growing HCT116 and LoVo cell tumours with 5-FU/oxaliplatin, TL32711 or the combination of both. As mathematically predicted for the in vivo setting, for both cell line models we indeed found significantly improved responses upon subjecting mice to the combination treatment with 5-FU/oxaliplatin and TL32711 compared to either 5-FU/oxaliplatin or TL32711 alone (Fig. 6c). cIAP1 expression was lost and cIAP2 expression decreased in tumour samples following TL32711-based treatments, confirming that the IAP antagonist was efficacious in vivo. (Fig. 6d). Interestingly, we found that cIAP2 expression was downregulated in both xenograft models following 5-FU/oxaliplatin treatment; however, co-treatment with TL32711 partially restored cIAP2 levels, potentially due to activation of non-canonical NF $\kappa$ B signalling following TL32711 treatment [8]. Most notably, expression of XIAP was reduced in both xenograft models following co-treatment with 5-FU/oxaliplatin and TL32711 (Fig. 6d).

Next, we examined how well the effects of the different treatments on reducing in vivo tumour growth would correlate with the treatment-specific predictions obtained from the mathematical model. We first determined the effects of the different treatments on tumour volume changes over time. We replicated experimentally measured tumour volume changes of individual mice by a growth impairment model, incorporating factors that represented maximal asymptotic growth and proliferation

rates. From this, we obtained the respective drug effects ( $K_D$ ) that contribute to the observed growth impairment [25, 26] (see also Methods). The tumour volume changes

could be fitted by the growth model, as seen by comparing pooled fitting data on volume changes to experimental data (Fig. 6e, f; Fig. 6c). When plotting cell death



◀ **Fig. 5** Systems modelling predicts increased apoptosis resistance of xenografted HCT116 and LoVo cell tumours, which can be overcome by chemotherapy/TL32711 co-treatment. **a** Comparison of baseline protein expression between in vitro and in vivo (day 7 after inoculation) growth conditions prior to treatment. For the in vivo setting, two xenografts were collected per cell line. Representative immunoblots from whole cell extracts of HCT116 and LoVo cells are shown. **b, c** For each cell line, protein expression amounts (nM) were quantified ( $n = 3-4$  detections) using quantitative immunoblotting. The apoptosis competency of ensembles of 1000 cells per treatment condition was mathematically modelled for HCT116 cells (**d**) or LoVo cells (**e**) for in vivo growth conditions

predictions against the drug effects obtained from the experimentally observed tumour volume changes (Fig. 6g), we found that the model predictions indeed correlated strongly with the observed in vivo treatment responses. These results confirmed that our mathematical model can predict in vivo responses of different CRC cell lines to standard of care chemotherapy, IAP antagonist TL32711 and the combination of both.

## Discussion

CRC is the second leading cause of cancer-related death in the Western world. In stage IV metastatic disease, median OS is only 21–24 months, 5-year OS rates are <6% and 10-year OS rates <1% [27]. IAP antagonists synergise with numerous anti-cancer drugs in various model systems [6], and several are currently in phase II clinical trials (<http://clinicaltrials.gov/>). TL32711 is in clinical trials for the treatment of advanced solid tumours, haematological malignancies and chronic hepatitis B and has been combined with the apoptosis-inducing human monoclonal antibody conatumumab in patients with relapsed epithelial ovarian cancer (NCT01940172). In a phase II clinical trial combining TL32711 and irinotecan in colorectal cancer patients refractory to irinotecan, the combination was tolerated similar to irinotecan alone resulted in clinical benefits particularly in patients with mutated *KRAS* (NCT01188499). However, patient selection strategies for IAP antagonists both as monotherapies and in combination with other agents are currently lacking.

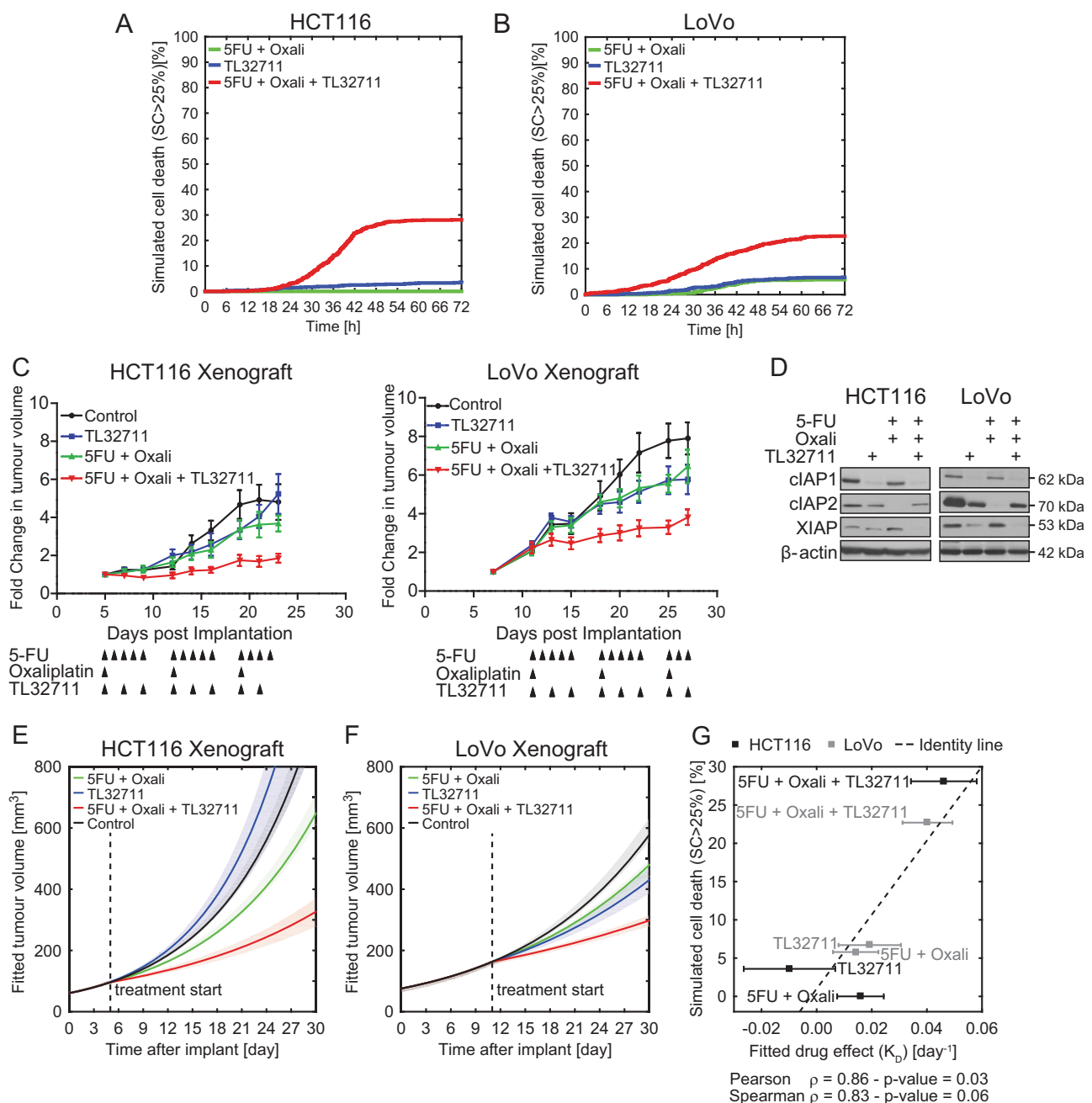
Having found that XIAP impairs chemotherapy-induced cell death in CRC cells, we mechanistically and quantitatively characterised the impact of TL32711 on chemotherapy-induced cell death in vitro and in vivo, taking into account cell-to-cell heterogeneity in isogenic cell populations. We developed a systems biology-based approach towards predicting treatment responsiveness to single and combination treatments of TL32711 with standard-of-care CRC chemotherapy in such cell populations. Results from the mathematical simulations correlated

robustly with the observed drug effects on xenograft tumour growth.

IAP antagonist-dependent degradation of cIAPs can induce intrinsic ripoptosome formation [28, 29], a finding we confirmed in CRC cells treated with TL32711. Interestingly, in contrast to work in fibrosarcoma, breast adenocarcinoma, rhabdomyosarcoma or ovarian carcinoma cell lines [16], chemotherapy alone was not sufficient to induce ripoptosome formation in CRC cells, but significantly enhanced the limited ripoptosome formation observed after TL32711 monotherapy. Depletion of procaspase-8 or RIPK1 abrogated cell death induced by TL32711/chemotherapy combinations. Our imaging data reveal that in response to TL32711/chemotherapy co-treatment, genetically identical cells differ in their mode of cell death induction, with both pre-MOMP caspase activation being observed (most likely due to ripoptosome formation), as well as subpopulations which trigger MOMP without prior caspase activation. We currently cannot answer where these response heterogeneities emerge from, but speculate that stochastic cell-to-cell heterogeneities in baseline protein expression might account for these differences. In addition, cell cycle asynchronies might contribute, since a number of apoptosis-regulating proteins are known to be cell cycle regulated and cells in different phases of the cell cycle will encounter different cell-cycle checkpoints upon chemotherapy-induced DNA damage [30]. The existing uncertainties regarding these crucial upstream signalling processes as well as the currently limited quantitative and kinetic knowledge of ripoptosome formation are the reasons why in our mathematical model, the upstream signalling was modelled from the behaviour of cell populations.

It has been reported that certain cell lines might secrete tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and die by autocrine TNFR1-dependent complex II formation. In our studies, neither HCT116 nor LoVo cells secreted TNF- $\alpha$  in response to TL32711 (not shown). However, we cannot exclude that the presence of TNF- $\alpha$  in tumour microenvironments further contributes to treatment efficacy. Indirect evidence for this emerges in the field of melanoma therapies, where it was recently described that the combination of TL32711 and chemotherapy does not induce response synergies in vitro, although single-treatment with TL32711 significantly impaired in vivo xenograft growth [31, 32]. While our systems model captures in vivo drug effects with very promising accuracy for single and combination treatments of TL32711 and CRC chemotherapy, the future inclusion of micro-environmental TNF- $\alpha$  signalling might improve the performance.

We believe that using mathematical simulations to predict in vivo efficacy of novel apoptosis-targeted agents such as IAP antagonists might have potential for contributing to the future personalisation and optimisation of treatments.



**Fig. 6** Mathematical modelling captures drug effects observed in vivo and predicts treatment responses. **a, b** Accumulated cell death as obtained from mathematically modelling apoptosis execution in HCT116 or LoVo cell ensembles at in vivo growth conditions. **c, d** Fold change in tumour volume in HCT116 and LoVo xenograft models in control, TL32711, 5FU and oxaliplatin and 5FU, oxaliplatin and TL32711 groups. Treatments of 15 mg/kg 5-FU, 2 mg/kg oxaliplatin and 30 mg/kg TL32711 were administered by IP injection as indicated. Error bars indicate s.e.m. **d** Western blot analysis of cIAP1,

cIAP2 and XIAP expression in HCT116 and LoVo xenograft tumours before and after treatment.  $\beta$ -actin served as loading control. **e, f** Pooled results from fitted tumour growth curves obtained from individual tumours. Traces indicate mean volume changes over time; shaded areas indicate variance (25th–75th percentile), obtained by bootstrapping, within the treatment group. **g** Correlation analysis between simulated cell death and the drug effects ( $K_D$ ) obtained from **e, f**. Error bars indicate s.d., obtained from bootstrapping

We recently demonstrated the added value of mathematically modelling apoptosis competency in combination with clinic-pathological markers for prognosticating disease outcome in chemotherapy-treated stage III CRC patients

[33]; in the future, similar approaches could be followed for chemotherapy/IAP antagonist therapies. Moreover, this basic framework could be extended to take account of the influence of tumour architectures, vascularisation,



microenvironmental heterogeneities as well as whole body drug PK/PD [34, 35]. More advanced experimental models, such as patient-derived xenografts, would provide ideal testing grounds for such model extension, refinement and optimisation.

In summary, this work provides proof-of-principle and in the future could possibly provide the basis for contributing to development of clinical tests in identifying colorectal cancer patients who are most likely to benefit from the addition of IAP antagonists to standard-of-care chemotherapeutics.

## Materials and methods

### Compounds and plasmids

TL32711 was obtained from Active Biochem (Maplewood, NJ). 5-FU (Medac) and oxaliplatin (Accord) were obtained from Belfast City Hospital. z-VAD-fmk was purchased from Merck Millipore (Darmstadt, Germany). Plasmids for the expression of the CFP-YFP FRET probes were described previously [17, 20].

### Cell lines

HCT116 and LoVo cell lines were purchased as authenticated stocks from ATCC (Teddington, UK). HCT116 XIAP<sup>-/-</sup> cells were provided by Dr. B. Vogelstein (Johns Hopkins University). HCT116 (Bax/Bak)<sup>-/-</sup> cells were kindly provided by Dr. R.J. Youle (National Institute of Neurological Disorders and Stroke).

### siRNA transfections

Scrambled control and RIPK1 siRNA (ON-TARGET SMART pool) were obtained from Dharmacon (Chicago, IL). Caspase-8 siRNA (5'-GAGUCUGUGCCCAAU-CAATT3') was purchased from Qiagen (Crawley, UK). siRNA transfections were carried out with Lipofectamine RNAiMAX transfection reagent (Invitrogen, Paisley, UK) according to manufacturer's instructions.

### Western blotting

Western blotting was carried out as previously described [21]. cIAP1, cIAP2 and caspase-8 antibodies were from Enzo (Exeter, UK). XIAP, caspase-3, SMAC, APAF-1, caspase-9 and cytochrome c antibodies were from Cell Signaling Technology (Danvers, MA).  $\beta$ -actin antibody was from Sigma (Missouri) and FLIP antibody from AdipoGen (San Diego, CA). Secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). Images were

captured using an Odyssey Imaging System (LICOR, Lincoln, NE) at 12 bit dynamic range. Quantification of protein expression amounts was conducted as described previously [21].

### Immunoprecipitation

Cells were pre-treated with 10  $\mu$ M z-VAD-fmk for 1 h prior to treatment with chemotherapy/TNF $\alpha$ /TL32711 combinations. Cells were lysed in CHAPS buffer (30 mM Tris pH7.5, 150 mM NaCl, 1% CHAPS). One microgram of caspase-8 p18 or IgG isotype control antibody (Santa Cruz, CA) was conjugated with 25  $\mu$ L ProteinG Dynabeads (Invitrogen, Paisley, UK). Protein lysate (750  $\mu$ g) was immunoprecipitated for 6 h at 4 °C. Ripoptosome formation was analysed by Western blotting for RIPK1 (BD Transduction Laboratories).

### Flow cytometry

Apoptosis induction was determined by Annexin V/propidium iodide analysis. Cells were harvested and stained according to the manufacturer's instructions (BD Biosciences, Oxford, UK). For measurements of mitochondrial membrane potentials, cells were incubated with 25 nM tetra-methyl-rhodamine ethyl ester (TMRE; Sigma, Missouri) for 15 min at 37 °C. Cells were then trypsinized, washed in PBS and analysed immediately. For FRET-based measurements of caspase-8 activation, Bax/Bak deficient HCT116 cells stably expressing the IETD FRET probe were generated and analysed as described before [18].

### Time lapse imaging and FRET analysis

Imaging settings and image analysis workflows for FRET disruption kinetics and for the timing of mitochondrial depolarisation at the single-cell level were described previously [17, 36]. All measurements were performed using stably transfected and clonally expanded cells. Cells were not synchronised prior to analysis to avoid potential cell cycle-dependent bias. Treatment durations exceeded two cell-cycle durations to obtain authentic data from otherwise unperturbed cell populations.

### Xenograft experiments

Female BALB/c nude mice were maintained as previously described [37]. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act, 1986 under project licence (PPL 2590b). Mice were implanted in each flank with  $2 \times 10^6$  HCT116 or  $2.5 \times 10^6$  LoVo cells in Matrigel (BD, Oxford, UK). Upon establishment of  $\sim 100$  mm<sup>3</sup> tumours (HCT116, day 5; LoVo, day 11), mice were



randomised to treatment groups. Groups were treated with vehicle, 5-FU/oxaliplatin, TL32711, or 5-FU/oxaliplatin in combination with TL32711. All treatments were administered by IP injection. TL32711 powder was reconstituted in 1% DMSO/peanut oil (Sigma, Missouri), and mice were injected with 30 mg/kg three times per week. 5-FU was administered at 15 mg/kg in PBS (Gibco) on a 5-day on/2-day off schedule. Oxaliplatin was administered at 2 mg/kg in H<sub>2</sub>O once a week. Tumour volume was assessed through measuring two dimensions using digital callipers three times per week. Two xenografts were collected per cell line from control mice to determine in vivo protein expression amounts.

## Mathematical modelling

MATLAB including Statistics Toolbox Release 2014b (The MathWorks., Cambridge, UK) was used for mathematically modelling apoptosis signalling in response to chemotherapy, TL32711 or the combination of both. For processes subsequent to MOMP, we employed APOPTO-CELL, an ordinary differential equations-based model previously validated to reliably simulate apoptosis execution [21, 38]. This model provides kinetics of CFP-DEVD-YFP FRET substrate cleavage as an output that can be compared to experiments in which such probes are used. To simulate the slow and modest substrate cleavage prior to MOMP (“pre-MOMP ramps”), the upstream signalling was modelled using a heuristic function that feeds into and triggers the execution phase model. The technical implementation is described in Supplemental Information S11.

## Correlation of model predictions and drug effects on tumour growth

Tumour growth inhibitory effects ( $K_D$ ) induced by the respective treatments were estimated from the in vivo tumour growth data. For this, we expressed the observed volume change over time ( $\frac{dV}{dt}$ ) from the difference between tumour growth when untreated and a treatment-mediated reduction in growth [25, 26]. Model-predicted cell death and drug effects on in vivo tumour growth ( $K_D$ ) were then tested for correlation (Pearson and Spearman). The technical implementation is described in Supplemental Information S11.

## Statistics

All statistical analyses were performed in Graphpad Prism (GraphPad Software, Inc., CA, USA). Data are shown as mean values  $\pm$  s.d., unless otherwise indicated. Two-tailed Student's *t*-tests were used for group comparisons; *p* values  $\leq 0.05$  were considered to be statistically significant.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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