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ORIGINAL ARTICLE

Stable XIAP knockdown clones of HCT116 colon cancer cells are more sensitive to TRAIL, taxanes and irradiation in vitro

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Abstract
Purpose To develop a model of X-linked inhibitor of apoptosis (XIAP) down regulation in colorectal cancer cell lines. This may be used to determine whether combination strategies have clinical potential.
Methods A series of clones were developed using short hairpin RNA (shRNA) against XIAP stably expressed in HCT116 cells. XIAP mRNA and protein levels were established by RT-PCR and Immunoblot, respectively. GeneChip microarrays confirmed XIAP knockdown and absence of compensation by other IAP members.
Results Four XIAP knockdown cell lines show 82–93% reduction in XIAP mRNA and 67–89% reduction in protein when compared to four luciferase control cell lines. XIAP knockdown sensitises cells to rhTRAIL by a factor of 3, to paclitaxel and docetaxel by a factor of >2 and, to a lesser extent, radiotherapy (20% enhancement).
Conclusions Clinical trials with XIAP antisense continue, and these data suggest combination studies with agents such as rhTRAIL and taxanes should be undertaken.

Keywords XIAP · HCT116 · TRAIL · Radiotherapy · Paclitaxel · Docetaxel · Microarray

Abbreviations

AMPK	AMP-activated protein kinase
cIAP1	Cellular inhibitor of apoptosis protein 1
cIAP2	Cellular inhibitor of apoptosis protein 2
DR	Death receptor
IAP	Inhibitor of apoptosis protein
NSCLC	Non-small cell lung cancer
NAIP	Neuronal apoptosis inhibitor protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RMA	Robust multichip average
RNAi	RNA interference
rhTRAIL	Recombinant human TNF related apoptosis inducing ligand
RT-PCR	Real time polymerase chain reaction
shRNA	Short hairpin RNA
SRB	Sulforhodamine B
XIAP	X-linked inhibitor of apoptosis protein

Introduction

Apoptosis results from activation of caspases in response to a wide variety of cell death stimuli via intrinsic and extrinsic pathways. Failure of cancer cell death and resistance to therapeutic treatments is partly due to failure to activate these pathways [1]. The X-linked inhibitor of apoptosis (XIAP) is a member of the inhibitor of apoptosis (IAP)

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family and the most potent endogenous caspase inhibitor [2]. The main function of XIAP is inhibition of executioner caspases 3 and 7 [3] and initiator caspase 9 [4], and XIAP is the only member of the family to inhibit both phases of the cascade directly [5]. It is debatable whether other members of the IAP group inhibit caspases, cIAP1 and cIAP2 have recently been shown to bind to but not inhibit caspases [6], questioning their role in the apoptotic pathway. Survivin has a role in cell division and is thought to stabilise XIAP by formation of an IAP-IAP complex [7]. More recently, an increase in XIAP levels was noted on detachment of intestinal epithelial cells from the extracellular matrix suggesting a role for XIAP in anoikis [8]. Schwab et al. [9] describe a decrease in XIAP when colorectal cancer cell lines were incubated with mesalazine suggesting a role for this drug in the chemoprevention of colorectal cancer in patients with inflammatory bowel disease. There are likely to be further roles which are as yet unidentified.

XIAP mRNA is over expressed in most of the panel of NCI 60 cell lines compared to normal cells suggesting a potential role as a therapeutic target in a wide spectrum of malignancies. XIAP mRNA is elevated in human prostate and lung tumours [10, 11], and in clinical colorectal tumour samples increases in XIAP protein levels have been found when compared to normal tissue [12]. XIAP has been associated with poorer prognosis in acute myeloid leukaemia [10] and renal cell carcinoma [13]. However, in cervical cancers, XIAP did not show any prognostic significance [14], and in radically resected NSCLC, high levels of XIAP were associated with increased survival and were inversely correlated with proliferation markers [15].

Two approaches are currently in development to clinically inhibit XIAP: antisense oligonucleotides and small molecule inhibitors [16]. XIAP antisense (Aegera Therapeutics Inc) is in Phase I trial [17] and antitumour activity has been identified as a single agent. Combinations of this targeted agent with other drugs may overcome resistance to current treatment regimes.

This study aimed to develop an isogenic model of XIAP in colon cancer cells with high specificity of target inhibition to investigate, both in vitro and in vivo, the effect of XIAP down regulation as a combination strategy with other treatment modalities in colorectal cancer.

Materials and methods

Chemicals and antibodies

All chemicals were from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Cytotoxics used were: Recombinant Human TRAIL/TNSF10 (R&D systems, Abingdon, UK Cat no 375-TEC), Paclitaxel 6 mg/ml (Bristol-Myers Squibb, New York, USA), Docetaxel (Sanofi-aventis, Guildford, UK). Primary antibodies used were: mouse XIAP monoclonal AAM-050 (Stressgen, Ann Arbor, Michigan, USA), mouse β-actin CP01 (Merck Biosciences Ltd, Nottingham, UK). Horseradish peroxidase-conjugated secondary antibodies for XIAP and beta-actin were from Autogen Bioclear UK Ltd, Calne, UK, and Merck Biosciences Ltd., respectively. DR5 primary antibody (ab18365) conjugated to phycoerythrin was from Abcam, Cambridge, UK.

Cell lines

HCT116 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 containing 2 mM glutamine, 5% foetal calf serum and 1% penicillin G/streptomycin. Cell cultures were incubated in the presence of 5% CO_2 at 37°C in a humidified atmosphere and split once a week with a solution of trypsin/EDTA (0.05%/0.02%).

Generation of stable XIAP shRNA HCT116 cells

XIAP shRNA plasmid constructs were kindly provided by Aegera Therapeutics Inc, Montreal, QC, Canada, according to the method described in [18] using a pCDNA3 vector containing a U6 promoter. Transfection with 1 μg of pCDNA_U6_luc (L prefix clones) or pCDNA_U6_shXIAP (X prefix clones) using Effectene (Qiagen, Crawley, UK) was performed according to manufacturers protocol. G418 (geneticin, Invitrogen, Paisley, UK) 50 mg/ml was used to select and maintain the vector expressing cells at 1:50 dilution.

RNA extraction and quantitative RT-PCR

Total RNA was extracted using Tri-Reagent followed by DNase treatment with 20 U of grade I DNase (Roche Applied Science, Mannheim, Germany) in the presence of 50 U RNase inhibitor. RNA was recovered after phenol/chloroform and chloroform extractions followed by precipitation with 3 M sodium acetate/absolute ethanol. Concentration was determined by spectrometry at 260 and 280 nm, and quality evaluated by electrophoretic analysis on a 2100 Agilent bioanalyser.

All transcripts were detected with QuantiTect SYBR Green RT-PCR kits (Qiagen) using 10 ng total RNA per reaction and primers at a concentration of 20 μM on a Rotor-Gene 3000 real-time DNA detection system (Corbett Life Science, Concorde, NSW, Australia).

Detection of XIAP protein by immunoblotting

A total of 25 μg protein was incubated with denaturing buffer (0.3 M Tris pH 6.8, 10% 2-mercaptoethanol, 40%

glycerol, 20% SDS, 0.02% bromophenol blue) for 5 min at 95°C, loaded onto a 10% SDS-polyacrylamide gel for electrophoresis, transferred onto polyvinylidene fluoride membranes, blocked in 5% non-fat milk TBS-Tween for 1 h at room temperature and incubated overnight at 4°C in a primary antibody against XIAP at 1:1,000 dilution. Membranes were also blotted against mouse beta-actin antibody 1:120,000 dilution as loading control. Membranes were then washed in TBS-Tween, and horseradish peroxidase-conjugated secondary antibodies incubated for 1 h at room temperature and washed again. Immunoreactivity was detected using the Enhanced ChemiLuminescence Plus detection reagent (GE Healthcare, London, UK) visualised on the Storm 840 Scanner (Amersham Biosciences, Amersham, UK). Protein quantitation was performed with Image Quant version 5.2 software.

Microarray study

A complete description of procedures and quality control measures is available at http://bioinformatics.picr.man.ac.uk/vice/ExternalReview.vice?k=7uzbaYssqNNuGUAUwv%2B893aw%2F%2FnR8cD%2FOwNgkjNgihRIysznCIlJuDkrKAX6K28URsPN%2FtSKupmC%0D%0Acu0smTolaA%3D%3D**.** Data were analysed using Bioconductor 1.8 [19] running on R 2.3.0 [20]. Expression measures were calculated using the 'Affy' package's Robust Multichip Average (RMA) default method [21]. Differential gene expression was assessed between X and L cell lines for early (p4) and late (p8) passage cells using an empirical Bayes' *t* test as implemented in the 'limma' package [22–24]. Subsequent *P* values were subjected to multiple testing correction using Benjamini and Hochberg's method for controlling the false discovery rate [25].

Cell growth and characteristics

Cells were seeded in 96-well plates at increasing densities (500–5,000 cells per well), and the number of cells estimated by sulforodamine B (SRB assay) [26] every 24 h in order to construct a growth curve for each cell line. The optimal cell density was defined as an optical density of >1.5 when the cells were allowed to grow for three doubling times. A graph was drawn of log cell number against time, the equation parameters for exponential growth are $y = \mathrm{No}.e^{kt}$. The doubling time was calculated according to the equation $t^2 = \ln 2/k$. The plating efficiency is the ratio between the numbers of cells present after 24 h when the media has been aspirated compared to no aspiration. The floating cell fraction was expressed as a percentage of the total adherent and floating cells and determined by counting aliquots using a coulter counter (Beckman Coulter Inc, Fullerton, CA, USA).

Flow cytometry with Annexin V-PI assay

Apoptotic cells were detected by flow cytometric analysis using TACS Annexin V-FITC kit (R & D systems, TA4638) according to manufacturer's instructions. Exponentially growing cells were analysed on the FACSCalibur (Benton Dickinson, San Jose, CA, USA) and instrument settings optimised using HCT 116 cells which were unstained, stained with Annexin V-FITC only or PI only. Identical gating was used for each cell line. Data analysis was performed with CellQuest software (BD Biosciences, San Jose, CA, USA).

In vivo xenograft establishment

Animal experiments were carried out under a project licence issued by the UK Home Office and UKCCCR guidelines [27] were followed rigorously. Studies were performed in Nu/Nu mice 3 months old bearing bilateral xenografts, five animals per group (10 xenografts). Ten million cells per flank were implanted and two dimensional volume measurements taken three times per week. Xenografts were collected on day 26, dissected to remove mouse tissue and blood vessels, and snap frozen in liquid nitrogen.

In vitro cytotoxicity

All experiments were performed at a passage of less than 8. Cells were exposed to rhTRAIL over a concentration range for 24 h prior to performing cytotoxicity studies with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay by incubation with 0.2 mg/ml MTT for 3 h in the dark at 37°C, removing media and solubilising formazan crystals in 200 µl DMSO. The optical density was measured at 570 nm using a BP800 microplate reader (Biohit, Helsinki, Finland). Paclitaxel and docetaxel were added over a concentration range to exponentially growing cells and incubated for 72 h prior to SRB assay [26]. A single fraction of γ radiation was applied over a dose range 0–16 Gy and cells incubated at 37°C for 120 h prior to SRB assay.

Caspase 3/7 activity induced by rhTRAIL

Cells were treated with rhTRAIL 5 ng/ml for 2.5 h and caspase 3/7 activity estimated by ApoOne caspase 3/7 assay (Promega, Madison, WI, USA) according to manufacturer's instructions. Fluorescence was detected on a Fluoroskan Ascent FL platereader (Thermo Labsystems, Waltham, MA, USA) using Ascent software version 2.4.1.

Statistical analysis

Statistical analyses of cytotoxicity data were performed using GraphPad Prism software version 4.00 for Windows,

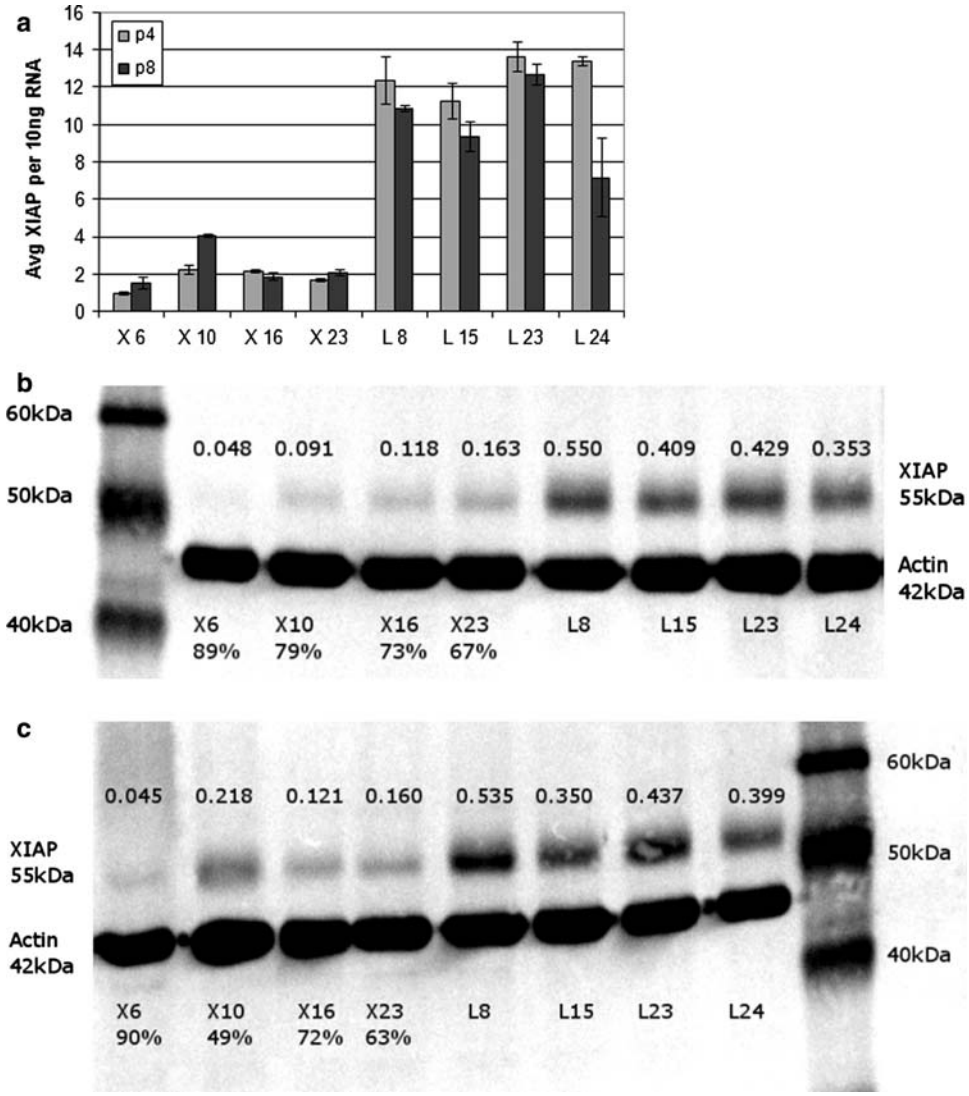

Fig. 1 XIAP knockdown at RNA and protein levels in luciferase expressing vector control (L prefix) and vector expressing short hairpin RNA to XIAP (X prefix). **a** Average XIAP mRNA levels per 10 ng total RNA by qRT-PCR at early (p4) and late (p8) passage of cells. *Error bars* show SD for triplicate samples in the experiment. XIAP protein expression determined at early passage (p4) (**b**) and late passage (p8) (**c**)

GraphPad Software, San Diego CA, USA. Data are expressed as mean values ± SD (unless otherwise stated); an unpaired two-tailed *t* test was used to detect significance. A value of $P < 0.05$ was considered to be statistically significant.

Results

Isolation of the cell lines and confirmation of IAP status

Twenty-four geneticin-resistant clones were isolated after transfection with shRNA expressing either XIAP (X clones) or luciferase (L clones). XIAP mRNA expression was determined by qRT-PCR and identified 4 X clones (X6, X10, X16 and X23) with low XIAP mRNA expression. Four L clones (L8, L15, L23 and L24) were selected with mean XIAP mRNA expression similar to the mean of the whole L group (11.6 ± 3.7). The mean expression of XIAP mRNA in the four selected vector control cell lines was 12.7 ± 1.1. The knockdown clones show reductions of 93, 82, 83 and 87% in XIAP mRNA for X6, X10, X16 and X23, respectively (Fig. 1a) relative to the mean of the XIAP levels in the vector expressing L control group. The mean level of XIAP mRNA of four selected clones in the L group (12.7 ± 1.1) was not significantly different to the level in the parental cell line (15.3 ± 2.0, *t* test $P = 0.07$). Stable knockdown at the mRNA level was maintained at passage 8 in X6 85%, X16 81% and X23 79% (Fig. 1a); however, the mRNA levels in X10 had risen to only 59% knockdown.

XIAP protein levels by Western immunoblot confirmed knock down in the X clones of 89, 79, 73 and 67% for X6, X10, X16 and X23, respectively relative to the mean level in four L clones (0.435 ± 0.08) (Fig. 1b). Stable knockdown was confirmed by protein quantitation at passage 8 (Fig. 1c) in X6 90%, X16 72% and X23 63%. These results are consistent with the early passage data described above; however, the protein levels in X10 had increased to only

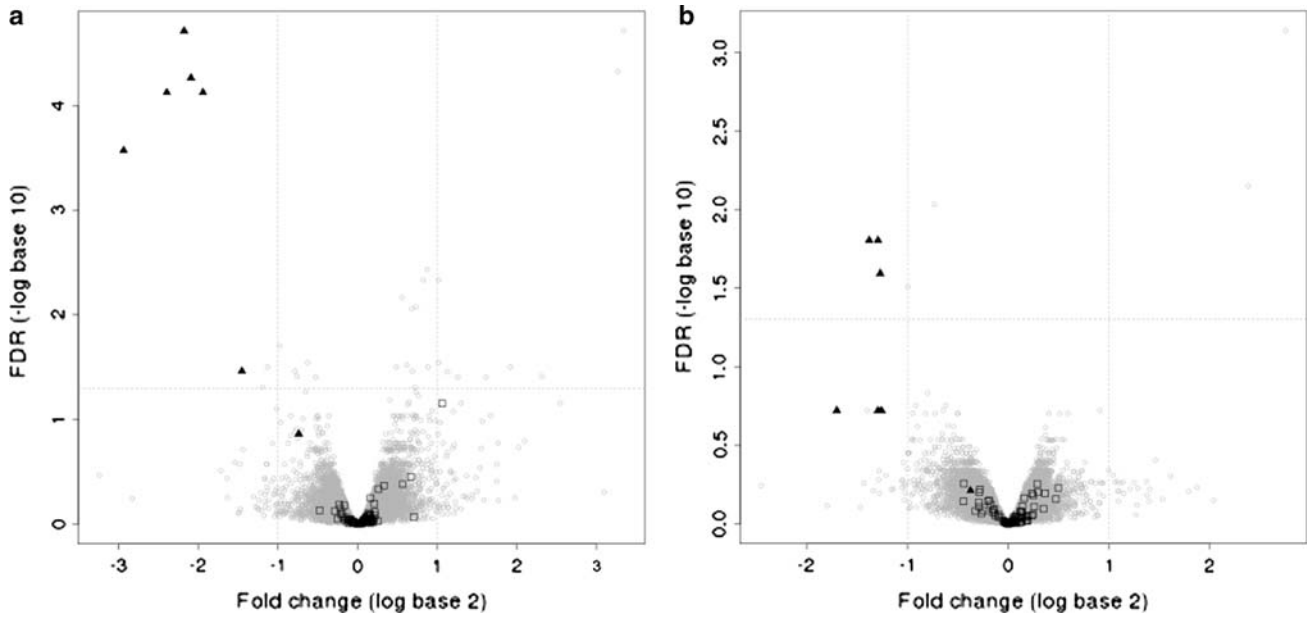

Fig. 2 Specific knockdown of XIAP only (*filled triangle*) with no impact on other members of the IAP family, bcl2 family, apoptotic pathway or factors reported as showing association with XIAP (*open square*). *Volcano plot* showing the fold change against adjusted *P* value for 4 X versus 4 L cell lines from RNA extracted from early passage cells p4 (**a**) and later passage cells p8 (**b**). X axis represents $\log_2$-fold change (*negative numbers* show down regulation in X relative to L cell lines and *positive numbers* show up-regulation), the *two vertical lines* represent ±2-fold change in expression. *Y* axis is a negative $\log_{10}$ transformation of the FDR (false discovery rate) adjusted *P* value (points above this line pass a *P* value cut-off of <0.05)

49% XIAP knockdown. Owing to the lack of long-term stability of the construct in the X10 cell line it was removed from cytotoxicity studies.

Microarray analysis confirms down regulation of XIAP RNA in the 4 X cell lines compared to the 4 L cell lines for seven different probe sets on the Affymetrix chip. The mean XIAP fold change is greater at the p4 time point 4.3 ± 1.9 when compared to the p8 time point 2.4 ± 0.6 (data not shown). The XIAP fold change at p4 is comparable to the down regulation seen by qRT-PCR, these are different techniques, but both assess changes at the mRNA level; there is good correlation between the two techniques.

Members of the IAP family cIAP1, cIAP2 and survivin were also analysed by qRT-PCR and the results compared with microarray data for the 4 X and 4 L cells lines relative to the parental HCT116 values. The R^2 values for these correlations are: XIAP 0.80–0.98, cIAP1 0.57, cIAP2 0.90–0.98, Survivin 0.90–0.94. There was good correlation between microarray and qRT-PCR data for three out of four members of the IAP family investigated. cIAP1 shows a poorer correlation, however, there is only one probe set for this gene and perhaps this highlights the need for multiple probesets on the GeneChip. In all cases there is no correlation of IAP family expression with XIAP status (data not shown).

The microarray data were analysed to identify potential differences in six other IAP family members (NAIP, cIAP1, cIAP2, survivin, apollon, livin), and no change in expression identified. The seventh member of this family (ILP2) was not represented on the Affymetrix chip. Members of the bcl-2 family (bcl-2, bax, bak1, bad, bcl-xl, bcl-w, bid) and apoptotic pathway members TRAIL, DR4, DR5, FAS, FADD, and caspases 3, 7, 8 and 9 also showed no differential expression in this model. Other factors which have been reported as showing interactions with XIAP on literature review (MURR1 [28], akt2 [29], smac/Diablo [30], XAF1 [31], htra2/omi [32], apaf1 [12], GSPT1 [33] and NFKB [34]) were unchanged (Fig. 2a, b). The above results confirm specific and stable 80% down regulation of XIAP with no impact on related genes.

In these data sets there are only six probe sets with significantly altered expression at both time points: XIAP represented three of these, one was on the borderline for fold change criteria, and two were from the same gene which was up regulated (PRKAA2). The protein encoded by PRKAA2 is a catalytic subunit of the AMP-activated protein kinase (AMPK) which is activated in response to cellular metabolic stress.

Cell line characteristics

The growth curves of the seven cell lines differed at a seeding density of 3,500 cells per well (Fig. 3a) with the X cell lines growing at a slower rate than the L cell lines. Thus to produce the superimposed growth curve necessary for cytotoxicity experiments a greater number of X cells per well

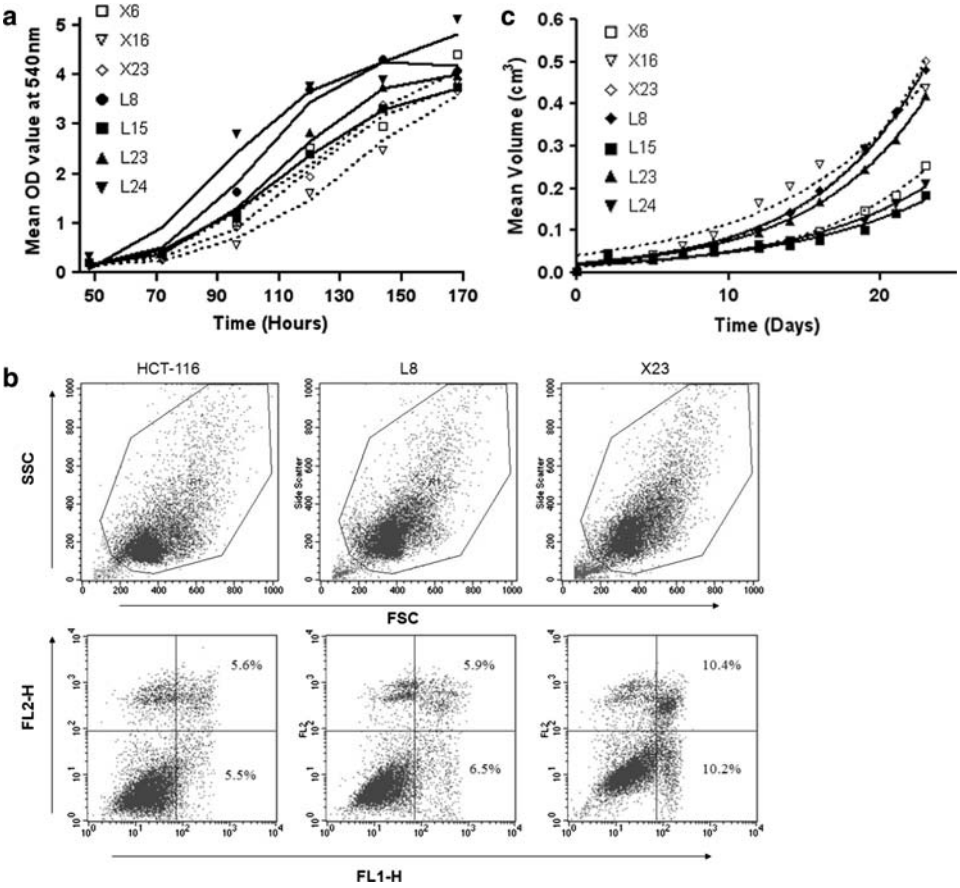

Fig. 3 Cell line characteristics. **a** Growth curve by SRB assay at seeding density of 3,500-cells per well. **b** Annexin V–PI staining in HCT116, L8 and X23 cells by flow cytometry. **c** Tumour growth rate of HCT116 XIAP knockdown cell lines and luciferase expressing controls in vivo. Results are expressed as mean volume (cm^3) for each group ($n = 10$)

were required; X6 4000, X16 5000, X23 5000 cells per well compared to L8 3500, L15 4000, L23 4000, L24 2500 L cells per well. When these seeding densities are adhered to similar doubling times are seen (mean 21.7 ± 1.36 h).

The plating efficiency varied across all cell lines examined; therefore, all experiments were designed to avoid removal of growth media and introduction of bias. The X cell lines have a higher floating cell fraction (1.11–3.42%) compared to the L cells lines (0.28–1.27%). The level of caspase activity in the cell lines was determined to investigate whether stable XIAP knockdown leads to increased activation of the apoptotic pathway, a late event. The levels of activated caspase 3/7 by ApoOne assay were unchanged when comparing L8 (57 ± 3.0 RFU) and X23 (46 ± 8.1 RFU), *t* test $P = 0.14$ (data not shown). Annexin V–PI staining showed a higher percentage of early apoptotic cells in the X23 cell line 10.2% compared to 6.5% in the L8 cell line (Fig. 3b). However, the increased fraction of cells which stained positive for both Annexin V and PI (10.4 vs. 5.9%) indicates that there was also a higher proportion of cells which have cell membrane damage, and therefore, these results should be interpreted with caution. The expression of TRAIL receptors (DR5) on the cell surface was quantified by flow cytometry prior to performing experiments with the agonist rhTRAIL. The median fluorescence was 30, 75, 91, 89 for Jurkat cells, HCT116, L8 and X23, respectively confirming that the receptor expression between the control cell line and XIAP knockdown cell line was unchanged (data not shown).

All eight of the vector containing cell lines were successfully established in vivo (Fig. 3c), although the growth patterns differed from the in vitro results described in Fig. 3a. L8 and L23 xenografts grew faster than L24 and L15 (which had a longer lag phase) and the X clones had intermediate growth rates. The eight cell lines could be divided into two groups with a shorter (L8, L23, X23 and X16) or longer phase before exponential growth (X6, L15 and L24). Therefore, one X and one L cell line were selected for further investigation to overcome these difficulties. L8 and X23 had similar growth curves in this model and were therefore selected as the pair of cell lines for chemotherapeutic studies in order to eliminate bias in comparison of cell growth patterns. RNA was extracted from two xenografts in each cell line and XIAP quantified after 26 days in vivo growth. The XIAP knockdown in vivo was maintained X6 76%, X16 67%, X23 46% when expressed as a proportion of mean XIAP level in the 4 L clones. This implies that the X cell lines continue to express the short hairpin RNA despite the absence of G418 selection medium although there is less XIAP down regulation when compared to the in vitro model.

Impact of XIAP down regulation on drug and radiation sensitivity

Cytotoxicity studies were performed with rhTRAIL to directly stimulate the extrinsic apoptotic pathway. X23 cells show a threefold increase in cytotoxicity compared to L8 (Fig. 4a); mean IC50 for X23 0.39 ng/ml (SD 0.16) compared to mean IC50 for L8 1.31 ng/ml (SD 0.42), $P < 0.05$ by t test. To confirm that loss of cell viability was due to the activation of the extrinsic apoptotic pathway, caspase 3/7 activity was estimated and a twofold increase in caspase 3/7 activity seen in the XIAP knockdown cell line; X23 186 RFU (SD 20.2) compared to L8 87 RFU (SD 19.7), $P < 0.004$ by t test (Fig. 4b).

Clinically, colorectal cancer is commonly treated with radiotherapy; therefore, we investigated the effect of this treatment modality in our XIAP knockdown model. The radiotherapy dose for 50% survival of the 3 X clones was 9.7, 11.5, and 10.2 Gy for X6, X16 and X23, respectively and 12.2, 14.3, 13.4, and 13.7 Gy for L8, L15, L23 and L24, respectively (Fig. 5a). XIAP knockdown therefore increased the sensitivity to radiotherapy by 20% (mean 50% survival value = 10.5 Gy (SD 0.93) for X clones and 13.4 Gy (SD 0.88) for L clones, $P < 0.02$ by t test).

Preliminary data from cytotoxicity experiments with 5FU, camptothecin and oxaliplatin where cells were treated at the IC_{50} concentration showed no benefit of XIAP down regulation across the X and L cell lines and therefore full cytotoxicity studies were not pursued.

The paclitaxel IC_{50} ranged from 5.0 in X6 to 5.9 nM in X16, and from 8.1 nM in L8 to 35.6 nM in L23 and from 1.7 nM in X23 to 2.8 nM in X16, from 5.9 nM in L8 to >50 nM in L23 for docetaxel. The three X cell lines were therefore on average twofold more sensitive than the L cell lines to paclitaxel and docetaxel (Fig. 5b, c). The mean IC_{50} for paclitaxel was 5.5 ± 0.35 nM and >7.9 nM for X and L cell lines, respectively; a similar increase in sensitivity was observed for docetaxel (mean IC_{50} 2.2 ± 0.56 nM for X cell lines and >5.9 nM for L cell lines). As the percentage cell death did not reach 50% in some of the L cell lines (L23, L24) at the highest concentration, an IC_{50} value could not be accurately calculated and this also precluded statistical evaluation.

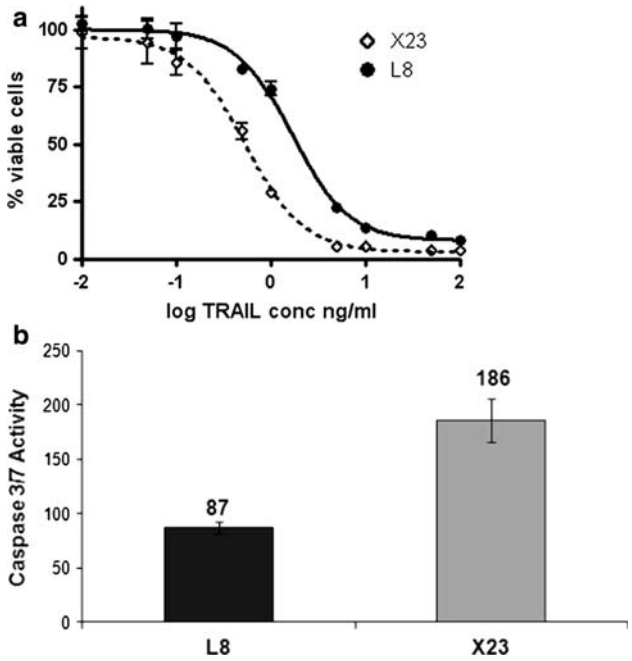

Fig. 4 Effects of XIAP knockdown on the sensitivity to rhTRAIL. Sensitivity of cells to 24-h exposure to rhTRAIL was determined by MTT assay (**a**) and caspase 3/7 assay (**b**)

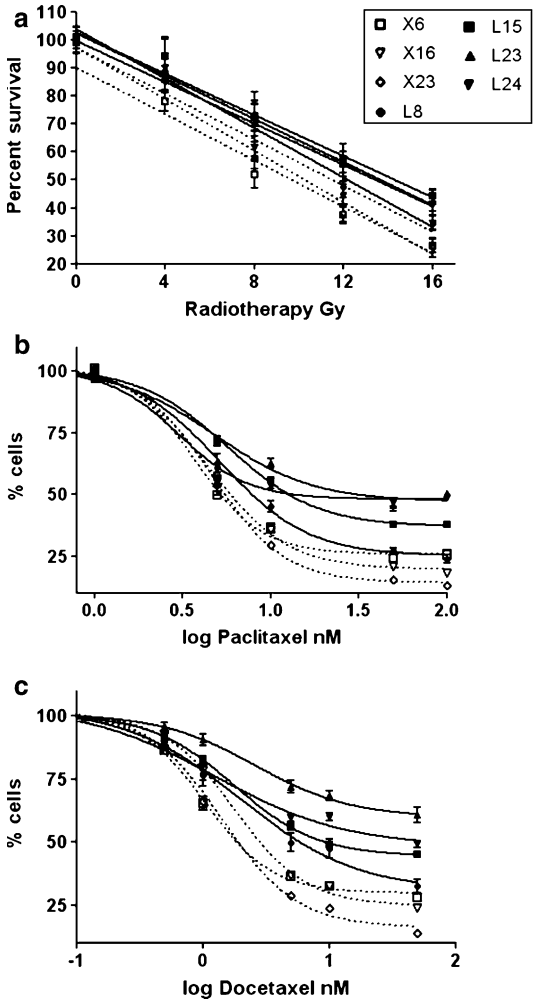

Fig. 5 Effects of XIAP knockdown on the sensitivity to γ radiation (**a**), paclitaxel (**b**) and docetaxel (**c**). Sensitivity to γ radiation (0–16 Gy), paclitaxel (1–100 nM) and docetaxel (0.5–50 nM) for 72 h were determined by SRB assay. *Error bars* show SEM for 3 individual experiments

Discussion

This study reports the development and characterisation of an isogenic model of XIAP knockdown developed from the HCT116 human colon cancer cell line. There is down regulation of 82–93% at the mRNA level which is generally stable (79–85%) over the course of four passages. The three XIAP knockdown cell lines developed here may therefore be confidently used for cytotoxicity studies in vitro over many weeks. Previous studies using the same vector construct expressing shXIAP induced 85% reductions in the mRNA level and 85–95% reductions in the XIAP protein level in the breast cell line MDA-MB-231 [18]. In a phase I clinical trial of XIAP Antisense (AEG35156, Aegera Therapeutics Inc), 82% knockdown of XIAP mRNA was seen in circulating lymphoma cells [17]. The level of XIAP down regulation achieved in this model of approximately 80% is similar to those achieved clinically.

There have been concerns that down regulation of XIAP would be compensated by increases in other members of the IAP family implying redundancy of the target. In the XIAP knockout mouse [35], there were increases in cIAP1 and cIAP2 protein levels and this may be the case in a model with complete XIAP loss rather than 80% down regulation. The results described above confirm specific and stable down regulation of XIAP with no impact on the related genes identified by other authors and confirmed in a previous study with the same vector construct [18].

One of the differences highlighted in our model is the growth characteristics of the shXIAP expressing cells. Previous experiments to validate XIAP as a target in colorectal cancer [36] have used clonogenic assays to demonstrate the effect of XIAP down regulation and these assays may detect growth delay rather than cell death by apoptosis. It is possible that stable down regulation of XIAP causes subtle phenotypic changes which have yet to be identified. When the XIAP knockout mouse was developed [35] there were initially thought to be no phenotypic changes, but since then differences in copper metabolism [28], mammary gland development [37] and signal transduction cascades [5] have been documented. This is to be expected, as it is now known that role of IAPs extend beyond caspase inhibition; there is evidence for effects on cell division, cell cycle progression and signal transduction [9, 38]. AMPK, the catalytic subunit of which was found to be up regulated in these XIAP knockdown clones, is thought to function as a suppressor of cell proliferation; [39] hence, providing a potential explanation for the growth delay in these cells although further studies would be required.

There have been reports of death receptor up-regulation following radiation treatment and also variation across colorectal cell lines [40]. The Death Receptor 5 (DR5) expression in the shXIAP cell lines was unchanged compared to vector control cells. There was no good correlation between expression of the DR5 receptor and cytotoxicity with the DR5 agonist monoclonal antibody [41].

Annexin V-PI assay results showed an increased early apoptotic fraction in the X23 cell line suggesting cells have commenced the journey along the apoptotic pathway, but the effectors of apoptosis are not yet activated as we report similar levels of caspase 3/7 activity across untreated X and L colorectal cell lines. Given that initial studies of XIAP described its ability to directly inhibit caspases [2], it was necessary to examine whether XIAP down regulation alone increased caspase activation. McManus et al. [18] found no increase in caspase 3 or 9 activity in untreated stable XIAP knockdown breast cancer cells consistent with our data. There have been reports of XIAP down regulation alone inducing apoptosis [42]; XIAP inhibition with antisense caused an increase in apoptosis in a lung cancer model in vitro which suggests that the effect may be due to the acute short-term nature of the knockdown.

The method of XIAP down regulation currently in clinical trial is XIAP antisense (AEG35156, Aegera Therapeutics Inc), which is delivered as a continuous infusion. The tissue half-life of the drug is known to be long (many days) though the plasma half-life is only a few hours. The model developed here therefore draws more parallels with the clinical situation where the goal is to cause XIAP tissue levels to decrease for a significant period of time as compared to transient transfection in cell lines models which may cause cellular changes related to the transfection agents used.

Several studies have developed isogenic systems to evaluate the biology of XIAP in different cell lines: Cummins et al. [36] were able to demonstrate a marked increase in sensitivity to TRAIL-mediated apoptosis and a corresponding decrease in clonogenic survival in a XIAP null HCT116 model, XIAP down regulation using RNAi in pancreatic and [43] breast cancer cells [18] demonstrated an increased sensitivity to TRAIL. Another approach using antisense oligonucleotides showed an increased sensitivity to ionising radiation in NSCLC cells [42] and to TRAIL in renal cancer cells [13]. In colorectal cancer, there is evidence for XIAP as a therapeutic target in vitro in combination with TRAIL [36, 44], in a mouse model of hepatic metastasis in vivo [45] and in clinical samples [29, 46]. TRAIL receptor antibodies have been shown to delay colorectal xenograft tumour growth [40] and are in early clinical development [47, 48].The shXIAP cells developed here are threefold more sensitive to TRAIL in vitro compared to vector control cells. A twofold increase in caspase 3/7 activity was demonstrated confirming that the increase in TRAIL cytotoxicity was due to activation of the apoptotic cascade. Our results therefore reproduce the effect described by Cummins et al. [36], though to a lesser degree, which may be

explained by the 80% knock down of XIAP compared with the complete knockout seen in the null system. In breast and pancreatic cancer cell lines [18], using the same short hairpin vector with similar levels of XIAP down regulation, a 100-fold increase in cytotoxicity was seen. We could therefore hypothesise that a greater decrease in XIAP levels is required in a colorectal cancer model.

A 20% increase in sensitivity to γ radiation was identified in the XIAP knockdown clones when compared to the controls. Marini et al. [40] noted that a single fraction of γ radiation induced less than 10% apoptosis in the HCT116 wild-type cell line; HCT116 was relatively resistant to radiotherapy compared to two other colorectal derivatives COLO205 and HCT15. Radiotherapy studies performed with antisense oligonucleotides to XIAP transiently transfected in vitro into non small cell lung cancer cell lines led to a 20% increase in apoptotic cells after treatment with 2-Gy radiation [42].

When treated with cytotoxic agents representative of those used in the clinical treatment of colorectal cancer (5FU, camptothecin and oxaliplatin), there was no significant difference when comparing three X with four L cell lines. In the XIAP null system, pilot experiments showed "small differences in cytotoxicity between XIAP null and wild-type HCT116 cells when treated with cytotoxic agents such as 5FU" (Personal communication F. Bunz) [36]. This implies that the sensitivity of these drugs is independent of XIAP in the HCT116 model.

Our data show at least a twofold increase in cytotoxicity of taxanes in the XIAP deficient cell lines. There is evidence that XIAP knockdown sensitises to taxane therapy in prostate and lung cancer models [49] and the data presented here would concur. In a breast cancer model using the same RNAi construct McManus et al. [18] were able to show sensitisation of approximately one order of magnitude in the XIAP deficient cell line which is consistent with our data. The effects seen with taxanes are more likely to be associated with the mechanism of action of taxanes rather than the characteristics of the tumour types. Paclitaxel has been shown to induce caspase 10 dependent apoptosis [50], which is a component of the extrinsic pathway (involving the TRAIL receptors DR4 and DR5). Taxanes are commonly used clinically in the treatment of prostate, lung and breast cancer but not colorectal cancer. However, with a relatively small increase in sensitivity in colorectal cancer models, it is unlikely these treatments will be of additional clinical benefit in patients with colorectal cancer.

In summary, four XIAP knockdown cell lines derived from HCT116 show 82–93% reduction in XIAP mRNA and 67–89% reduction in protein when compared to four luciferase control cell lines. Microarray analysis confirms XIAP down regulation at two time points and shows no compensation of genes known to be related to XIAP; the only additional gene which is significantly up regulated is PRKAA2. XIAP knockdown sensitises cells to rhTRAIL by a factor of 3, to paclitaxel and docetaxel by a factor of >2 and, to a lesser extent, radiotherapy (20% enhancement).

A Phase 1 clinical trial is underway in solid tumours combining XIAP Antisense (Aegera Therapeutics Inc) and docetaxel (National Cancer Institute of Canada Clinical Trials Group). Future work should also focus on targeted agents such as rhTRAIL which directly stimulate the extrinsic apoptotic pathway. One such agent, a death receptor agonist, is in early clinical trial [48], and may show promise in combination with strategies to down regulate XIAP.

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