Published in final edited form as:
Mol Cancer Ther. 2012 August ; 11(8): 1789–1798. doi:10.1158/1535-7163.MCT-11-0535.

Chemosensitisation of cancer cells by KU-0060648; a dual inhibitor of DNA-PK and PI-3K

Joanne M. Munck[1], **Michael A. Batey**[1], **Yan Zhao**[1], **Helen Jenkins**[3], **Caroline J. Richardson**[2], **Celine Cano**[2], **Michele Tavecchio**[1], **Jody Barbeau**[1], **Julia Bardos**[3], **Liam Cornell**[1], **Roger J. Griffin**[2], **Keith Menear**[3], **Andrew Slade**[3], **Pia Thommes**[4], **Niall MB Martin**[3], **David R Newell**[1], **Graeme CM Smith**[4], and **Nicola J Curtin**[1,*]

[1]Newcastle University, Northern Institute for Cancer Research, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK

[2]Newcastle University, Northern Institute for Cancer Research, School of Chemistry, Bedson Building, Newcastle upon Tyne, NE1 7RU, UK

[3]KuDOS Pharmaceuticals, Ltd., 410 Cambridge Science Park, Milton Road, Cambridge, CB4 0PE, UK

[4]AstraZeneca Oncology iMed, Mereside, Alderley Park, Macclesfield, SK10 4TG, UK

Abstract

DNA double strand breaks (DSBs) are the most cytotoxic lesions induced by topoisomerase II poisons. Non-homologous end joining (NHEJ) is a major pathway for DSB repair and requires DNA-PK activity. DNA-PKcs is structurally similar to PI-3K, which promotes cell survival and proliferation and is up-regulated in many cancers. KU-0060648 is a dual inhibitor of DNA-PK and PI-3K *in vitro.*

KU-0060648 was investigated in a panel of human breast and colon cancer cells. The compound inhibited cellular DNA-PK auto-phosphorylation with IC_{50} values of 0.019 μM (MCF7 cells) and 0.17 μM (SW620 cells), and PI-3K-mediated AKT phosphorylation with IC_{50} values of 0.039 μM (MCF7 cells) and > 10 μM (SW620 cells). 5-day exposure to 1 μM KU-0060648 inhibited cell proliferation by > 95% in MCF7 cells, but only by 55% in SW620 cells. In clonogenic survival assays, KU-0060648 increased the cytotoxicity of etoposide and doxorubicin across the panel of DNA-PKcs-proficient cells, but not in DNA-PKcs-deficient cells, thus confirming that enhanced cytotoxicity was due to DNA-PK inhibition. In mice bearing SW620 and MCF7 xenografts, concentrations of KU-0060648 that were sufficient for *in vitro* growth inhibition and chemosensitisation were maintained within the tumour for at least 4 hours at non-toxic doses. KU-0060648 alone delayed the growth of MCF7 xenografts and increased etoposide-induced tumour growth delay in both in SW620 and MCF7 xenografts by up to 4.5-fold, without exacerbating etoposide toxicity to unacceptable levels.

The proof of principle *in vitro* and *in vivo* chemosensitisation with KU-0060648 justifies further evaluation of dual DNA-PK and PI-3K inhibitors.

[*]Corresponding Author: Newcastle University, Northern Institute for Cancer Research, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK n.j.curtin@ncl.ac.uk Tel: +44 (0) 191 246 4415 Fax: +44 (0) 191 246 4301 .

Conflicts of Interest: NJC, RJG and DRN – receipt of research funding from KuDOS/Astrazeneca.
KM, HJ, CJR, NMBM, AS and JB – former employees of KuDOS
PT and GCMS – currently employed by AstraZeneca

Europe PMC Funders Author Manuscripts

Keywords

DNA-PK; DNA-PK inhibitor; PI-3K; PI-3K inhibitor; topoisomerase II poison chemosensitisation; cell survival; pharmacokinetics; antitumour activity/xenograft studies; human colon cancer

Europe PMC Funders Author Manuscripts

INTRODUCTION

DNA double strand breaks (DSBs) are the principal cytotoxic lesions induced by topoisomerase II poisons, such as etoposide and doxorubicin (1), which are widely used in the treatment of a variety of human solid malignancies and leukaemia (2). Unrepaired DSBs trigger cell cycle arrest and/or cell death, and it is thought that even a single DSB may be enough to induce cell death (1, 3).

As DSBs may also be generated endogenously or by environmental toxins, cells have developed complex mechanisms to repair DSBs for survival. In mammalian cells homologous recombination (HR) and non-homologous end-joining (NHEJ) are the major DSB repair pathways (4, 5). The principal factor determining the choice of mechanism is in which cell cycle phase the DSBs occur, or are recognised (6, 7). HR is dependent on the proximity of the sister chromatid and can therefore only take place in late S and G2 phase. In contrast, NHEJ rejoins broken DNA ends without reference to a second template and is predominant during G1 or G0 phase, but also operational during other cell cycle phases (8).

DNA DSB repair, whilst essential for survival, may constitute a mechanism of therapeutic resistance to certain DNA damaging agents. Consequently, cells defective in DSB repair by virtue of the inactivation of a component of the NHEJ pathway are highly sensitive to ionising radiation (IR) and topoisomerase II poisons (9-16). Conversely, over-expression of DNA-PKcs, a key component of the NHEJ pathway, can accelerate the repair of IR, etoposide and doxorubicin-induced DSBs, thus conferring resistance to these agents (17, 18). In the clinical setting, DNA-PK protein expression correlates with resistance to etoposide in human CLL samples (19). Therefore, the inhibition of DNA-PK is an attractive approach for modulating resistance to therapeutically-induced DNA DSBs.

DNA-PK is a member of the phosphatidylinositol-3 kinase (PI-3K)–related protein kinase (PIKK) family of enzymes. PI-3K regulates a wide range of cellular processes, including those central to cell growth and survival. Aberrant activation of the PI-3K signalling pathway is common in many human cancers (20-22). Therefore, the inhibition of PI-3K is also an attractive target for the development of cancer therapies.

Consistent with the structural similarities between PI-3K and DNA-PKcs, the PI-3K inhibitor LY294002 was identified as a competitive inhibitor of DNA-PK (23). LY294002 has been reported to retard DSB repair and enhance the cytotoxicity of IR, which despite the lack of specificity, has largely been attributed to inhibition of DNA-PKcs (24). Ongoing efforts within our research group using LY294002 as a starting pharmacophore for chemistry, gave rise to the discovery of more potent and specific inhibitors of DNA-PKcs (15, 25). Our group identified NU7441 as a potent inhibitor of DNA-PK (26-28), and continuing compound development has led to the identification of KU-0060648 2-(4-ethylpiperazin-1-yl)-*N*-(4-(2-morpholino-4-oxo-4*H*-chromen-8-yl)dibenzo[*b,d*]thiophen-1-yl)acetamide, which has >500-fold greater solubility, greater potency against DNA-PK and is a dual DNA-PK and PI-3K inhibitor, but shows selectively over other PI-3K-like kinases, such as ATM, ATR and mTOR. Here we describe the pre-clinical evaluation of KU-0060648.

Europe PMC Funders Author Manuscripts

Using cell-based assays, we demonstrate that KU-0060648 inhibits DNA-PK with 8-fold greater potency in MCF7 than SW620 cells and has at least a 50-fold greater potency against PI-3K in MCF7 than SW620 cells. In a panel of human cancer cell lines KU-0060648 (at a concentration of 1 μM) exhibited differential effects on growth inhibition, but was not profoundly cytotoxic. However, at this concentration it substantially enhances the cytotoxicity of the topoisomerase II poisons etoposide and doxorubicin in all cells studied. We demonstrate that the potentiation of etoposide and doxorubicin cytotoxicity by KU-0060648 is due to DNA-PK inhibition using DNA-PKcs-deficient and complemented cell lines. Furthermore, KU-0060648 enhances the anti-tumour activity of etoposide in both MCF7 and SW620 xenograft models, and has single-agent activity in the MCF7 xenograft model.

MATERIALS AND METHODS

Chemicals

KU-0060648 was provided by Dr Marc Hummersone, KuDOS Pharmaceuticals (KuDOS Horsham, UK). ZSTK474 was synthesised as previously described (29). Etoposide phosphate was purchased from the National Health Service (UK). All other chemicals were purchased from Sigma (Poole, UK), unless stated otherwise. For *in vitro* assays, KU-00606048, ZSTK474, doxorubicin and etoposide were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stocks and stored at −20 °C. For *in vivo* experiments KU-0060648 was dissolved in equimolar phosphoric acid, pH 5.

Cell lines and culture

LoVo and SW620 human colon cancer cells and T47D, MCF7 and MDA-MB-231 human breast cancer cell lines were obtained from the ATCC (Manassas, Virginia, USA). V3 (DNA-PKcs-deficient Chinese hamster ovary cells) and their derivative V3-YAC cells (transfected with a yeast artificial chromosome carrying the human DNA-PKcs gene), were a gift from Professor Penny Jeggo (University of Sussex, UK). M059J (DNA-PKcs-deficient human glioblastoma cells) (30) and their derivative M059-Fus-1 cells (transfected with human chromosome 8, carrying the DNA-PKcs gene) were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). All other cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). All human cells were authenticated by STR profiling (LGC Standards, Teddington, UK) within the last 2 months. Cells were free of *Mycoplasma* contamination (MycoAlert Assay, Cambrex Bio Science, Nottingham, UK) and were maintained at 37 °C in an atmosphere of 5% CO_2. V3-YAC and M059-Fus-1 cells were maintained under G-418 sulphate (Invitrogen, Carlsbad, CA) selection, at a concentration of 500 μg/ml and 200 μg/ml, respectively.

Determination of cellular activity of KU-0060648 against DNA-PK and PI-3K

DNA-PK autophosphorylation was determined in cells exposed to a range of concentrations of KU-0060648 for 1 hr prior to X-irradiation (10 Gy). Cell lysates were prepared 30 minutes later using Phosphosafe Extraction Reagent (Merck, Nottingham, UK) according to the manufacturer's instructions. Levels of DNA-PKcs auto-phosphorylation at Ser^{2056} (31) relative to un-phosphorylated DNA-PKcs were determined by western blotting. To determine PI-3K activity, cells were exposed to a range of concentrations of KU-0060648 for 1 hr prior to a 30-minute treatment with 50 ng/ml insulin-like growth factor-1 (Calbiochem, Merck Biosciences, Nottingham, UK). The levels of PI-3K-dependent AKT phosphorylation (Ser^{473}) (32) relative to un-phosphorylated AKT were determined by western blotting.

Gel electrophoresis was performed with Tris-Acetate 3-8% (v/v) polyacrylamide gradient gels (Bio-Rad, Hemel Hempstead, UK). Samples, transferred onto Amersham Hybond C nitrocellulose membrane (GE Healthcare Life Sciences, Buckinghamshire, UK), were probed with primary antibodies against DNA-PKcs (H-163; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Ser^{2056} DNA-PKcs (ab20407; 1:1000; Abcam, Cambridge, UK), AKT (C67E7; 1:500; New England Biolabs, Hitchin, UK), phosphorlyated Ser^{473} AKT (193H12; 1:500; New England Biolabs, Hitchin, UK) and actin (Ab-1; 1:10000; Calbiochem, Merck Biosciences, Nottingham, UK). Anti-rabbit or anti-mouse (actin) horseradish peroxidase–linked secondary antibodies (1:1000; Dako, Ely, UK) and ECL reagent (GE Healthcare Life Sciences, Buckinghamshire, UK) were used for detection, followed by image acquisition and densitometry using a Fuji LAS-3000 luminescent image analyzer (Raytek, Sheffield, UK).

The increase in phosphorylated protein above the baseline levels of un-stimulated cells was measured, and the level detected in extracts from KU-0060648-treated cells expressed as a percentage of the increase in control cells. The mean values of three independent experiments were plotted as a sigmoidal dose-response curve and the IC_{50} value calculated using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Cytotoxicity and Growth inhibition Studies

Cytotoxicity was measure by clonogenic assays. Cells grown in 6-well plates were exposed to etoposide or doxorubicin, with or without KU-0060648 (1 μM) for 16 hours, prior to harvesting and seeding into 10 cm diameter Petri dishes, in drug-free medium. Colonies were stained with crystal violet after 10 to 14 days and counted with an automated colony counter (ColCount, Oxford Optronics Ltd., Oxford, UK). Cell growth inhibition following 5-day continuous exposure to KU-0060648 was determined by SRB assay, as described previously (33). The GI_{50} is the concentration causing 50% cell growth inhibition.

KU-0060648 plasma pharmacokinetics following different routes of administration

All *in vivo* experiments were reviewed and approved by the relevant institutional animal welfare committees and performed according to national law. We determined the plasma pharmacokinetics of KU-0060648 following administration intravenously (i.v.), intra-peritoneally (i.p.) or orally (p.o.) at 10 mg/kg to female Balb C mice (Charles River, Ramsgate, UK). KU-0060648 was formulated in a vehicle of equimolar phosphoric acid, made up to volume with sterile saline and at final pH 5. Mice were killed at intervals up to 360 minutes after KU-0060648 administration and plasma concentrations of KU-0060648 were determined by LC-MS/MS analysis, as previously described(27).

KU-0060648 distribution to tumour xenografts

Female athymic mice (CD1-nu/nu Charles River) were maintained and handled in isolators under specific pathogen free conditions for tissue distribution and efficacy studies. KU-0060648 (12.5 mg/kg i.v.) was administered to MCF7 or SW620 tumour-bearing mice (650 mm^3), which were killed 60 or 240 minutes later. Tumours were excised and homogenised in PBS (1:3 w/v), using a stirrer mascercarator homogenizer (IKA® Werke GmbH & Co., KG. Germany), in 10 second bursts, on ice. Plasma and tumour KU-0060648 concentrations were determined by LC-MS/MS analysis, as previously described (27).

DNA-PK *ex vivo* pharmacodynamic assay

KU-0060648 at 2.5 or 25 mg/kg or vehicle alone was administered to SW620 tumour-bearing mice i.v.. After 1 or 4 hours, animals were killed and tumours were excised and homogenised. DNA-PK activity within tumour homogenates was determined by measuring

the DNA-PK-dependent phosphorylation of a p53 peptide substrate (Ser^{15}), using an ELISA assay, as described previously (34).

Anti-tumour efficacy study

Mice bearing SW620 or MCF7 xenografts s.c. (n = 5 per group) were treated when tumours were palpable (approx 5 mm × 5 mm, 8-10 days post implantation). Animals received normal saline i.p. once daily (control), single agent KU-0060648 10 mg/kg i.p. twice daily for either 5 days (2 × d × 5) in SW620 tumour-bearing, or 14 days (2 × d × 14) in MCF7 tumour bearing mice with doses on each day 8 hours apart, or etoposide phosphate once daily i.p. (11.35 mg/kg in saline, equivalent to 10 mg/kg free etoposide, i.p., d × 5). For combinations, KU-0060648 was administered i.p. once or twice daily for 5 days (SW620) or once daily for 14 days (MCF7), with the first dose immediately prior to etoposide phosphate.

Tumour volume was calculated from two-dimensional electronic caliper? (Mitutoyo, Andover, UK) measurements using the equation $a^2 \times b/2$ where a is the smallest measurement and b the largest. Data are presented as median relative tumour volume (RTV), where the tumour volume on the initial day of treatment (day 0) is assigned an RTV value of 1.

RESULTS

Cellular Activity of KU-0060648 against DNA-PK and PI-3K

We used a cell-based assay to determine the inhibitory activity of KU-0060648 against DNA-PK and PI-3K in MCF7 and SW620 cells. IR treatment induced an approximately 20-fold increase in DNA-PK auto-phosphorylation levels, which was inhibited by KU-0060648 in a concentration-dependent manner, with an IC_{50} value of 0.02 μM in MCF7 cells and 0.2 μM in SW620 cells (Supplementary Figure 2). IGF-1 treatment caused an approximately 8-fold increase in AKT phosphorylation, which in MCF7 cells was inhibited in a KU-0060648 concentration-dependent manner, with an IC_{50} value of 0.04 μM. In contrast, the previous lead inhibitor, NU7441, was approximately 20 times more potent against DNA-PK (IC_{50} = 0.2 ± 0.03 μM) than PI-3K (IC_{50} = 3.9 ± 3.5 μM) in MCF7 cells. KU-0060648 was virtually inactive against PI-3K in SW620 cells in which the IC_{50} for the inhibition of AKT phosphorylation was >10 μM (with only 42% inhibition at this concentration; Supplementary Figure 2), suggesting that the inhibition of PI-3K by KU-0060648 may be cell line-dependent. In a control experiment the PI-3K inhibitor ZSTK474 [2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine] (35) was used ot demonstrate that it is possible to inhibit AKT Ser^{473} phosphorylation using a PI-3K inhibitor in SW620 cells, Unlike KU-0060648, the compound did not cause such pronounced cell specific differences in inhibition of AKT phosphorylation, with IC_{50} values of 0.2 μM in SW620 cells and 0.02 μM in MCF7 cells (Supplementary figure 3).

Chemopotentiation by KU-0060648 *in vitro*

We investigated the DNA-PK-dependence of chemosensitisation by KU-0060648 in mammalian and human cells with and without DNA-PK function. The DNA-PKcs-deficient V3 cells were approximately 3-fold more sensitive to etoposide than the DNA-PKcs-proficient V3-YAC cells (Figure 1A and B). Similarly, the DNA-PKcs-deficient M059J cells were approximately 2.5-fold more sensitive to etoposide than the DNA-PKcs-proficient M059-Fus-1 cells (Figure 1C and D). KU-0060648 alone caused < 15% reduction in cell viability in V3 and V3-YAC cells and < 5% reduction in cell viability in M059J and M059-Fus-1 cells, but enhanced the cytotoxicity of etoposide by > 13-fold in V3-YAC cells and 4-fold in M059-Fus1 cells, compared with only 2.5-fold and 1.1- to 1.5-fold in V3 and M059J

cells, respectively (Figure 1A-D). There was an even more profound difference in the sensitivities of M059J and M059-Fus-1 cells to doxorubicin. M059J cells were approximately 23-fold more sensitive to doxorubicin than M059-Fus-1 cells. KU-0060648 enhanced the cytotoxicity of doxorubicin by up to 32-fold in M059-Fus-1 cells (depending on the doxorubicin concentration), but only 1.4-fold in M059J cells (Figure 1E and F). We confirmed that M059J and M059-Fus-1 cells have comparable PI-3K activity levels and that AKT phosphorylation is inhibited to the same extent by KU-0060648 and ZSTK474 in each of the cell lines (Figure 1G). Therefore chemosensitisation of topoisomerase II poisons by KU-0060648 is largely due to DNA-PK inhibition.

We investigated growth inhibition by KU-0060648 in a panel of human breast (MCF7, T47D and MDA-MB-231) and colon (LoVo and SW620) cancer cells. DNA-PK expression was confirmed in each of the cell lines and inhibition of DNA DSB repair by KU-0060648 was demonstrated in SW620 cells (Supplementary Figure 4). Exposure to 1 μM KU-0060648 for 5 days resulted in > 50% inhibition of cell growth in all cell lines (Figure 2A). The greatest effect on growth inhibition was observed in LoVo and MCF7 cells, in which total cell growth over 5 days was only 10% and 4% of that of DMSO treated controls, respectively. KU-0060648 had GI_{50} values of 0.95 μM in SW620, 0.21 μM in LoVo, 0.27 μM in MCF7, 0.41 μM in T47D and 1 μM in MDA-MB-231.

In comparison to the profound growth inhibition, the cytotoxicity of KU-0060648 was much less marked. Following a 16 hr exposure to 1 μM KU-0060648, overall cell survival was ≥ 80% in each of the cell lines except for MDA-MB-231 cells, which had a survival rate of 41% (Figure 2A). KU-0060648 (1 μM) markedly enhanced the cytotoxicity of doxorubicin and etoposide in all cell lines (Figure 2B and Table 1). In SW620 cells, KU-0060648 enhanced the cytotoxicity of 1 μM etoposide by over 100-fold and enhanced the cytotoxicity of 10 nM doxorubicin by over 10-fold. However, in LoVo, MCF7 and T47D cells the enhancement of doxorubicin cytotoxicity (50-100 fold) was greater than that of etoposide (< 10-fold). The lowest potentiation of etoposide or doxorubicin cytotoxicity by KU-0060648 was observed in MDA-MB-231 cells, in which a 3-4 fold sensitisation was observed.

Plasma pharmacokinetics of KU-0060648 following different routes of administration

The plasma pharmacokinetic parameters determined after administration of 10 mg/kg KU-0060648 to Balb/C mice by various routes are given in Table 2. The percentage bioavailability of KU-0060648 following p.o administration was found to be ≥100% The pharmacokinetic parameters of KU-0060648 following i.p. administration were found to be similar to that when given i.v., with 78% bioavailability.

Tissue distribution of KU-0060648 in MCF7 and SW620 tumour-bearing mice following i.v. administration

Following administration of KU-0060648 (12.5 mg/kg i.v.) to mice bearing either MCF7 or SW620 xenografts, KU-0060648 distributed extensively to the tumour and was retained after clearance from the plasma (Table 2). Concentrations of KU-0060648 of over 1 μM (a level resulting in chemosensitisation *in vitro*) were maintained in the tumour for at least 4 hours.

DNA-PK activity determined *ex vivo* in SW620 tumour samples

In order to investigate whether levels of KU-0060648 achieved within tumours were sufficient to have a biological effect, we determined DNA-PK activity by measuring DNA-PK-dependent phosphorylation of a p53 peptide substrate (Ser^{15}). Comparison of tumour samples taken from control animals or animals treated with 2.5 or 25 mg/kg i.v. KU-0060648 revealed a dose-dependent reduction of p53 (Ser^{15}) phosphorylation. Parallel

measurement of KU-0060648 PK revealed that tumour concentrations of KU-0060648 following a 25 mg/kg i.v. dose were sufficient to give almost 100% inhibition of DNA-PK activity for at least 4 hours (Figure 3).

Anti-tumour activity of etoposide and KU-0060648 in SW620 or MCF7 tumour-bearing mice

We investigated the antitumour activity of KU-0060648 against both MCF7 and SW620 xenografts. Mice bearing MCF7 tumour xenograftswere treated with etoposide phosphate, alone and in combination with KU-0060648 (Figure 4A). Tumours in control mice reached 4 times their starting volume (RTV4) at a median time of 10 days (Mean time to RTV4 = 9.6 ± 1.9 days). Treatment with etoposide phosphate alone caused a median growth delay of 38 days (time to RTV4 = 48 days. Mean time to RTV4 = 31.7 ± 8.6 days P = 0.0001). Treatment with KU-0060648 alone caused a median growth delay of 30 days (time to RTV 4 = 40 days. Mean time to RTV4 = 43.3 ± 3.6 days P= 0.0467), and combination of treatments caused a median growth delay of 55 days (time to RTV4 = 65 days. Mean time to RTV4 = 52 ± 6.8 days P = 0.006). The toxicity of KU-0060648 and etoposide phosphate alone was negligible (maximum body weight loss 3%) and the combination of drugs did not cause unacceptable toxicity (maximum body weight loss = 7%). In theSW620 xenograft model, tumours in control mice reached RTV4 at a median time of 5.5 days and consistent with *in vitro* studies KU-0060648 alone did not cause any tumour growth delay (P = 0.4573). Treatment with etoposide phosphate alone caused a median tumour growth delay of 1 day (time to RTV4 = 6.5 days), which was extended to 3.5 days (time to RTV4 = 9 days) by once daily co-administration of KU-0060648.When KU-0060648 was administered twice daily, a growth delay of a further 2 days (time to RTV4 = 11 days) was observed. However, none of the tumour growth delays were statistically significant due to the rapid growth of SW620 tumours and requirements to kill mice with large tumours, resulting in a reduced sample size.Neither KU-0060648 nor etoposide phosphate alone caused any significant toxicity (no body weight loss), and the combination of drugs did not cause unacceptable toxicity (maximum body weight loss = 11%).

DISCUSSION

Both DNA-PK and PI-3K, by virtue of their role in DSB repair and the promotion of cell proliferation, respectively, are attractive targets for the development of novel anti-cancer therapies. KU-0060648 is a sub micromolar inhibitor of DNA-PK and PI-3K in MCF7 cells, with a 10-fold reduced potency against DNA-PK and >250-fold reduced potency against PI-3K in SW620 cells. The potency in intact cells was substantially lower than in the cell-free assay (Supplementary Figure 1). As KU-0060648 is an ATP-competitive inhibitor, the discrepancy may be attributable to high intracellular ATP concentration. The approximately 10-fold difference in IC_{50} values for KU-0060648 between MCF7 and SW620 cells might reflect different cellular ATP concentrations between cell lines or cell-specific differences in uptake or efflux, resulting in higher concentrations within the MCF7 cells. Although neither hypothesis has been tested experimentally, inhibition of PI-3K-dependent AKT phosphorylation by ZSTK474 (control compound) was also 10-fold less potent in SW620 cells compared to MCF7 cells, and this could also be dependent on potential differences in uptake and ATP concentrations between the two cell lines. In further support of this hypothesis, lower levels of KU-0060648 were observed in SW620 tumour xenografts than MCF7 tumour xenografts, although the difference was not statistically significant (Table 2B). However, even accounting for a potential difference in KU-0060648 accumulation within cells, the lack of PI-3K inhibition by KU-0060648 in SW620 cells was striking. This suggests that KU-0060648 may inhibit PI-3K in a cell-line dependent manner. Alternatively, the complete lack of AKT phosphorylation inhibition in response to KU-0060648 compared with the dose-dependent response to ZSTK474 in the SW620 cells may reflect differential

Europe PMC Funders Author Manuscripts

effects of the compounds on the PI-3K isoforms and their relative activities in SW620 cells Of the four PI-3K isoforms, KU-0060648 has the greatest potency against PI-3K δ and the least potency against PI-3K γ (Supplementary Figure 1). Conversely, ZSTK474 has the greatest potency against PI3K γ and the least potency against PI-3K δ (36).

Consistent with the well-established role of PI-3K in cell proliferation (20, 37), KU-0060648 caused substantial growth inhibition across a panel of human cancer cell lines. The degree of growth inhibition was cell line-specific. The apparent lack of PI-3K inhibition by KU-0060648 in SW620 cells might explain why a 5-day exposure to KU-0060648 (1 μM) only conferred 50% growth inhibition in SW620 cells, compared with > 95% growth inhibition in MCF7 cells. Similarly, KU-0060648 was found to have single agent activity in mice bearing MCF7 xenografts (causing almost as much tumour growth delay as etoposide alone, at the doses studied), but had no single agent activity in mice bearing SW620 xenografts. In addition to the differential effects on AKT phosphorylation, a further explanation for the different effects of KU-0060648 on the growth of SW620 and MCF7 xenografts is that MCF7 cells (unlike SW620) harbor a PIK3CA mutation, and there is evidence that PIK3CA mutations confer sensitivity to single agent PI-3K inhibitors (38, 39). Consistent with these studies, we demonstrate that KU-0060648 conferred greater *in vitro* growth inhibition in the breast cancer cell lines MCF7 and T47D, which also harbor a PIK3CA mutation, than MDA-MB-231 cells.

The differential effects of KU-0060648 on *in vitro* growth inhibition across the panel of human cancer cell lines, and the effect of KU-0060648 on tumour growth delay in MCF7 xenograft-bearing mice, suggest that KU-0060648 may be effective as a single-agent therapy. The sensitivity to KU-0060648 as a single-agent appears to be largely cell line-dependent. However, characterisation of the molecular determinants for the sensitivity of cancer cells to KU-0060648, potentially PIK3CA mutations, may lead to the establishment of predictive biomarkers, which could be used to identify patients sensitive to KU-0060648 as a single-agent therapy.

Studies in isogenically paired DNA-PKcs-proficient and deficient cells (that have comparable levels of PI-3K activity, which was similarly inhibited by KU-0060648) confirm that KU-0060648 potentiation of etoposide and doxorubicin cytotoxicity in these models is largely due to DNA-PK inhibition. Substantial enhancement of etoposide and doxorubicin cytotoxicity was also observed across a panel of human cancer cell lines. However, there was significant cell line variation in the degree of enhancement. MDA-MB-231 cells were relatively resistant to sensitisation to both drugs (< 4-fold enhancement of toxicity). In MCF7 cells, KU-0060648 increased doxorubicin cytotoxicity > 100-fold, but only increased etoposide cytotoxicity 9-fold. Conversely, KU-0060648 increased doxorubicin cytotoxicity 12-fold and etoposide cytotoxicity >100-fold in SW620 cells. The differential chemosensitisation of two similar-acting drugs by KU-0060648 does not seem to be related to the p53-status of the cells (MCF7 and LoVo have wild type p53 (40, 41) and SW620 MDA-MB-231 and T47D have mutant p53 (42-44), but may depend on other molecular determinants in the cell panel that are yet to be determined

KU-0060648 had significant single agent anti-tumour activity that was comparable to that of etoposide in mice bearing MCF7 tumour xenografts, and the combination was more active than either drug alone. Neither KU-0060648 nor etoposide had any discernible effect on the growth of SW620 xenografts, but there was a modest trend towards increased antitumour activity by the combination of the two drugs, particularly when KU-0060648 was administered twice.

Overall, these studies demonstrate that KU-0060648 is a potent dual inhibitor of DNA-PK and PI-3K, which inhibits cell growth and enhances the cytotoxicity of topoisomerase II posions in a cell line-dependent manner. KU-0060648 showed good oral bioavailability and pharmacokinetics, resulting in concentrations within the tumour that are commensurate with cellular DNA-PK and PI-3K inhibition, *in vitro* cell growth inhibition and *in vitro* chemosensitisation. Furthermore, KU-0060648 conferred complete inhibition of DNA-PK activity in SW620 tumours. The differential sensitivities of SW620 and MCF7 xenografts suggest that there may be a tumour-specific response to KU-0060648, which highlights the need to identify predictive biomarkers, such as PIK3CA, for the selection of patients that are most likely to respond to KU-0060648. These data provide excellent “proof of principle” evidence that improvements in the anti-tumour activity of doxorubicin and etoposide are achievable through the dual inhibition of PI-3K and DNA-PK.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

NJC, CC, RJG and DRN gratefully acknowledge the financial and scientific support of KuDOS Pharmaceuticals and AstraZeneca.We are grateful to Marc Hummersone for the provision of KU-00060648 and Professor Penny Jeggo for giving us the V3 and V3-YAC cells

The work reported in this manuscript was conducted with the financial support of AstraZeneca and KuDOS Pharmaceuticals, Grant number AZ305642 awarded to NJ Curtin and DR Newell Cancer Research UK, Grant number C240/A7409 awarded to DR Newell, RJ Griffin, C Cano and NJ Curtin

References

1. Iliakis G. The role of DNA double strand breaks in ionising radiation-induced killing of eukaryotic cells. Bioessays. 1991:13.
2. Pommier Y, Leteurtre F, Fesen MR, Fujimori A, Bertrand R, Solary E, et al. Cellular determinants of sensitivity and resistance to DNA topisomerase inhibitors. Cancer Invest. 1994; 12:530–42. [PubMed: 7922710]
3. Ward J. The yield of DNA double-strand breaks produced intracellularly by ionising radiation: a review. Int J Radiat Biol. 1990; 57:1141–50. [PubMed: 1971840]
4. Jackson SP. Sensing and repairing DNA double strand breaks. Carcinogenesis. 2002; 23:687–96. [PubMed: 12016139]
5. Kanaar R, Hoeijmakers JH, van Ghent DC. Molecular mechanisms of DNA double-strand break repair. Trends in Cell Biology. 1998; 8:483–9. [PubMed: 9861670]
6. Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, et al. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO Journal. 1998; 17:5497–508. [PubMed: 9736627]
7. Yoshida M, Hosoi Y, Miyachi H, Ishii N, Matsumoto Y, Enomoto A, et al. Roles of DNA-dependent protein kinase and ATM in cell cycle-dependent radiation sensitivity in human cells. International Journal of Radiation Biology. 2002; 78:503–12. [PubMed: 12065055]
8. Rothkamm K, Kruger I, Thompson LH, Lobrich M. Pathways of DNA double-strand break repair during the mammalian cell cycle. Molecular Cell Biology. 2003; 23:5706–15.
9. Blunt T, Finnie NJ, Taccioli GE, Smith GC, Demengeot J, Gottlieb TM, et al. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. Cell. 1995; 80:813–23. [PubMed: 7889575]
10. Caldecott K, Banks G, Jeggo P. DNA-double strand break repair pathways and cellular tolerance to inhibitors of topoisomerase II. Cancer Research. 1990; 50:5778–83. [PubMed: 2168280]

11. Jeggo PA, Caldecott K, Pidsley S, Banks GR. Sensitivity of Chinese hamster ovary mutants defective in DNA repair to topoisomerase II inihibitors. 1989; 49:7057–63.
12. Jeggo PA. DNA breakage and repair. Adv Genet. 1998; 38:185–211. [PubMed: 9677708]
13. Lees-Miller SP, Godbout R, Chan DW, Weinfeld M, Day RS, Barron GM, et al. Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. Science. 1995; 267:1183–5. [PubMed: 7855602]
14. Tanaka T, Yamagami T, Oka Y, Nomura T, Sugiyama H. The scid mutation in mice causes defects in the repair system for both double-strand DNA breaks and DNA cross-links. Mutation Research. 1993; 288:277–80. [PubMed: 7688088]
15. Veuger SJ, Curtin NJ, Richardson CJ, Smith GCM, Durkacz BW. Radiosensitization and DNA Repair Inhibition by the Combined Use of Novel Inhibitors of DNA-dependent Protein Kinase and Poly(ADP-Ribose) Polymerase-1. Cancer Research. 2003; 603:6008–15. [PubMed: 14522929]
16. Smith G, Jackson SP. The DNA-dependent protein kinase. Genes and Development. 1999; 13:916–34. [PubMed: 10215620]
17. Hansen LT, Lundin C, Helleday T, Poulsen HS, Sørenson CS, Petersen LN, et al. DNA repair rate and etoposide (VP16) resistance of tumour cell subpopulations derived from a single human small cell lung cancer. Lung Cancer. 2003; 40:157–64. [PubMed: 12711116]
18. Shen H, Schultz M, Kruh GD, Tew KD. Increased expression of the DNA-dependent protein kinase confers resistance to adriamycin. Biochem Biophys Acta. 1998; 1381:131–8. [PubMed: 9685611]
19. Eriksson Å , Lewensohn R, Larsson R, Nilsson A. DNA-dependent protein kinase in leukaemia cells and correlation with drug sensitivity. Anticancer Research. 2002; 22:1787–94. [PubMed: 12168870]
20. Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002; 296:1655–7. [PubMed: 12040186]
21. Franke TF. PI3K/Akt: getting it right matters. Oncogene. 2008; 27:6473–88. [PubMed: 18955974]
22. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. Oncogene. 2008; 27:5497–510. [PubMed: 18794884]
23. Izzard RA, Jackson SP, Smith GCM. Competitive and noncompetitive inhibtion of the DNA-dependent protein kinase. Cancer Research. 1999; 59:2581–6. [PubMed: 10363977]
24. Rosenweig KE, Youmell MB, Palayoor ST, Price BR. Radiosensitization of human tumour cells by the phosphatidyl inositol 3-kinase inhibitors wortmanin and LY294002 correlates with inhibition of DNA-depepndent protein kinase and prolonged G2-M delay. Clinical Cancer Research. 1997; 3:1149–56. [PubMed: 9815794]
25. Willmore E, de Caux S, Sunter NJ, Tilby MJ, Jackson GH, Austin CA, et al. A novel DNA-dependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of luekaemia. Blood. 2004:4659–65. [PubMed: 15010369]
26. Hardcastle IR, Cockroft X, Desage El-Murr M, Leahy JJ, Stockley M, Golding BT, et al. Discovery of potent chromen-4-one inhibitors of the DNA-dependent protein kinase (DNA-PK) ising a small-molecule library approach. J Med Chem. 2005; 48:7829–46. [PubMed: 16302822]
27. Zhao Y, Thomas HD, Batey MA, Cowell IG, Richardson CJ, Griffin RG, et al. Preclinical evaluation of a potent novel DNA-dependent protein kinase (DNA-PK) inhibitor, NU7441. Cancer Research. 2006; 66:5354–62. [PubMed: 16707462]
28. Leahy JJ, Golding BT, Griffin RJ, Hardcastle IR, Richardson C, Rigoreau L, et al. Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. Bioorg med Chem Lett. 2004; 14:6083–7. [PubMed: 15546735]
29. Shinichi, Y.; Koshimizu, I.; Yoshimi, H.; Matsuno, T.; Watanabe, T.; Tsuchida, Y., et al. Treatment of prostate cancer, melanoma or hepatic cancer. U.S.A.: 2007. inventor Zenyaku Kogyo Kabushiki Kaisha, assignee.
30. Tsuchida R, Yamada T, Takagi M, Shimada A, Ishioka C, Katsuki Y, et al. Detection of ATM gene mutation in human glioma cell line MO59J by a rapid frameshift/stop codon assay in yeast. Radiation Research. 2002; 158:195–201. [PubMed: 12105990]

31. Chen BP, Chan DW, Kobayashi J, Burma S, Asaithamby A, Morotomi-Yano K, et al. Cell cycle dependence of DNA-dependent protein kinase phosphorylation in response to DNA double strand breaks. Journal of Biological Chemistry. 2005; 280:14709–15. [PubMed: 15677476]
32. Guillard S, Clarke PA, Te Peele R, Mohri Z, Bjerke L, Valenti M, et al. Molecular pharmacology of phosphatidylinositol 3-kinase inhibition in human glioma. Cell Cycle. 2009; 8:443–53. [PubMed: 19177002]
33. Skehan P, Storeng R, Scuderio D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxcitic assay for anticancer-drug screening. J Natl Cancer inst. 1990; 82
34. Griffin RJ, Fontana G, Golding BT, Guiard S, Hardcastle IR, Leahy JJ, et al. Selective Benzopyranone and Pyrimido[2,1-*a*]isoquinolin-4-one inhibitors of DNA-dependent Protein Kinase: Synthesis, Structure-Activity Studies, and Radiosensitization of a Human Tumour Cell Line in vitro. Journal of Medicinal Chemistry. 2005; 48:569–85. [PubMed: 15658870]
35. Yaguchi S, Fukui Y, Koshimizu I, Yoshimi H, Matsuno T, Gouda H, et al. Antitumour Activity of ZSTK474, a New Phosphatidylinositol 3 - Kinase Inhibitor. Journal of the National Cancer Institute. 2006; 98:545–55. [PubMed: 16622124]
36. Kong DaY T. ZSTK474 is an ATP-competitive inhibitor of class I phosphatidylinositol 3 kinase isoforms. Cancer Science. 2007; 98:1638–42. [PubMed: 17711503]
37. Nicholson KM, Anderson NG. The protein Kinase B/AKT signalling pathway in human malignancy. Cellular Signalling. 2002; 14:381–95. [PubMed: 11882383]
38. Brachmann SM, Hofmann I, Schnell C, Fritsch C, Wee S, Lane H, et al. Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and PI3KCA mutant breast cancer cells. Proc Natl Acad Sci USA. 2009; 106:22299–304. [PubMed: 20007781]
39. O'Brien C, Wallin JJ, Sampath D, GuhaThakurta D, Savage H, Punnoose EA, et al. Predictive biomarkers of sensitivity to the phosphatidylinositol 3′kinase inhibitor GDC-0941 in breast cancer preclinical models. Clinical Cancer Research. 2010; 16:3670–83. [PubMed: 20453058]
40. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, et al. The COSMIC (Catalog of somatic mutations in cancer) database and website. British Journal of Cancer. 2004; 91:355–8. [PubMed: 15188009]
41. Concun N, Zeillinger C, Tong D, Stimpfl M, Konig M, Printz D, et al. Comparison of p53 mutational status and protein expression in a panel of 24 human breast carcinoma cell lines. Breast Cancer Research and Treatment. 2003; 79:37–46. [PubMed: 12779080]
42. Bartek J, Iggo R, Gannon J, Lane DP. Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene. 1990; 5:893–9. [PubMed: 1694291]
43. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hosteller R, Cleary K, et al. Mutations in the p53 gene occurr in diverse human tumour types. Nature. 1989; 342:705. [PubMed: 2531845]
44. Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, et al. p53 mutations in colorectal cancer. Proc Natl Acad Sci USA. 1990; 87:5.

Europe PMC Funders Author Manuscripts

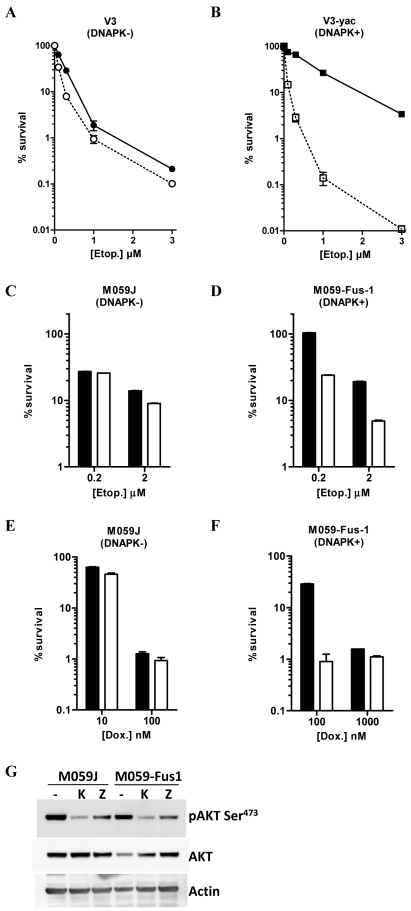

Figure 1. Determination of the cellular specificity of KU-0060648 for DNA-PK-dependent cell survival following exposure to etoposide or doxorubicin

A-B; Clonogenic survival of V3-yac and V3 cells exposed to etoposide alone (solid symbols) or in combination with 1 μM KU-0060648 (open symbols), for 16 hours prior to seeding for colony formation. C-D; Clonogenic survival of M059-Fus-1 and M059J cells exposed to etoposide alone (black bars) or in combination with 1 μM KU-0060648 (white bars), for 16 hours prior to seeding for colony formation. E-F; Clonogenic survival M059-Fus-1 and M059J cells exposed to doxorubicin alone (black bars) or in combination with 1 μM KU-0060648 (white bars), for 16 hours prior to seeding for colony formation. Data are the means ± SD of 3 independent experiments. G; M059J and M059-Fus-1 cells were exposed to 1 μM KU-0060648 (K) or 0.1 μM ZSTK474 (Z) for 1 hour. Cell lysates were prepared and the relative levels of PI-3K-dependent AKT Serine^{473} phosphorylation determined by western blot.

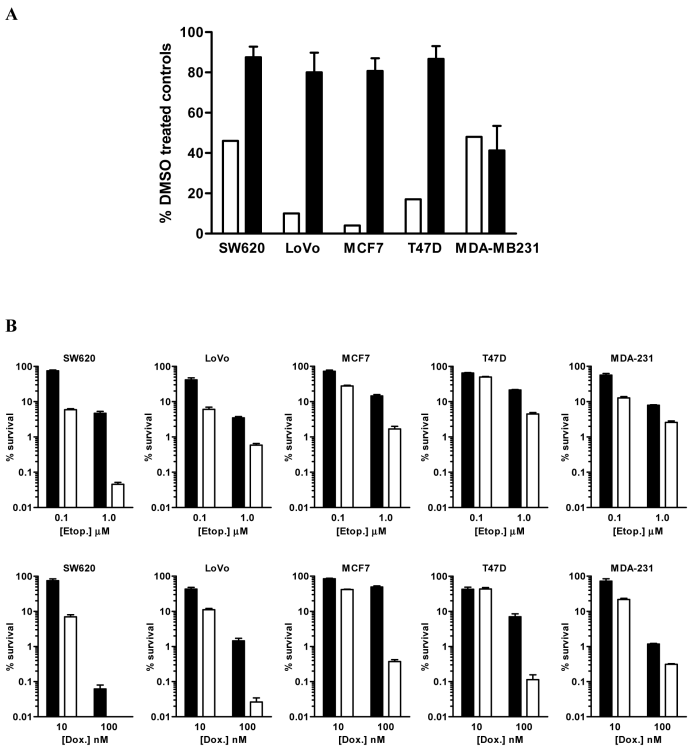

Figure 2. Growth inhibition, cytotoxicity and chemosensitisation of KU-0060648 in human colon and breast cancer cell lines

A. The effect of KU-0060648 on cell growth (white bars), following 5-day continuous exposure to KU-0060648 was determined as previously described [28]. Cytotoxicity of KU-0060648 (black bars) was determined by the clonogenic survival of cells exposed to KU-0060648 (1 μM) for 16 hours prior to seeding for colony formation. Data are the means ± SD of 3 independent experiments.

B. Clonogenic survival of cells exposed to etoposide or doxorubicin in the presence or absence of KU-0060648 (1 μM) for 16 hours, prior to seeding for colony formation. Topoisomerase II poison alone (black bars), Topoisomerase II poison + KU-0060648 (white bars). Data are the means ± SD of 3 independent experiments.

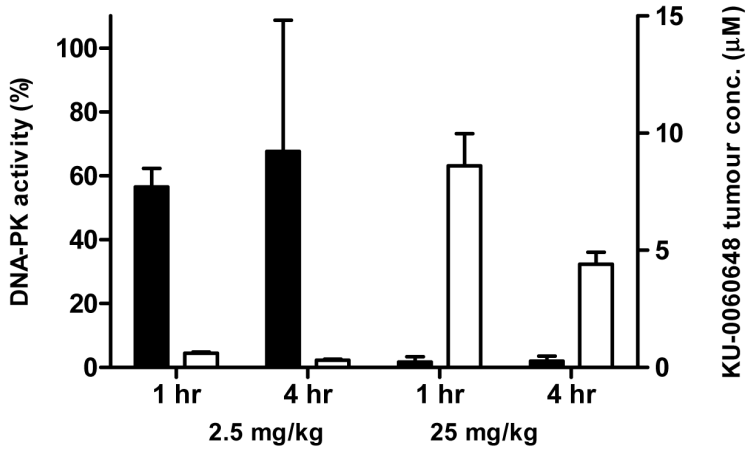

Figure 3. Comparison of PK levels of KU-0060648 and DNA-PK activity within *ex vivo* SW620 tumour samples following i.v dosing

CD-1 athymic female mice bearing SW620 human tumour xenografts were treated with KU-0060648 at either 2.5 or 25mg/kg i.v. At 1 or 4 hours following compound administration, plasma and tumour tissue were taken. KU-0060648 concentration (white bars) was measured by HPLC and the level of DNA-PK activity (black bars) was determined by measuring the DNA-PK-dependent phosphorylation of a p53 peptide substrate using an ELISA assay. Data are the mean ± SD of 3 replicate mice per time point.

Europe PMC Funders Author Manuscripts

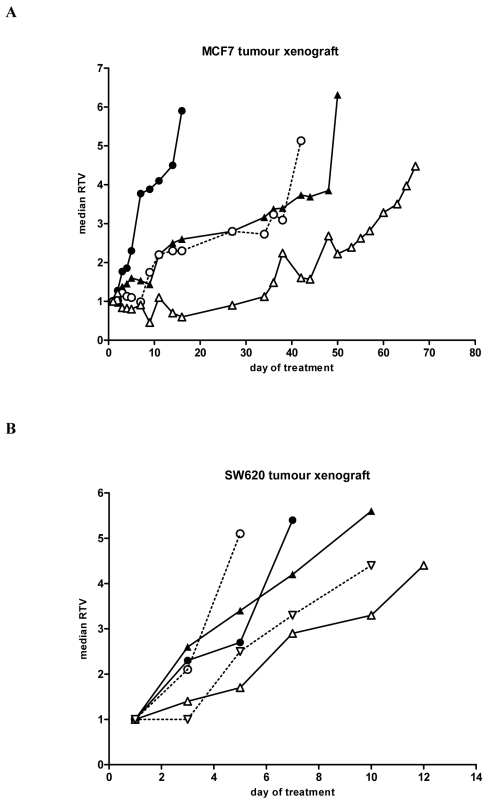

Figure 4. Efficacy of etoposide and KU-0060648 in MCF7 and SW620 sub-cutaneous xenografts

Growth of xenografts is presented as the median relative tumour volume (RTV). Treatment commenced when tumours were palpable (approx. 5mm × 5mm).

A. Animals bearing MCF7 tumours (5 /group) were treated with vehicle control (●), KU-0060648 alone (10 mg/kg twice daily × 14, ○), etoposide phosphate alone 11.5 mg/kg, daily × 5 , ▲) or KU-0060648 and etoposide (Δ).

B. Animals bearing SW620 tumours (5 /group) were treated with vehicle control (●), KU-0060648 alone (10 mg/kg daily × 5, ○), etoposide phosphate alone (11.5 mg/kg, daily × 5, ▲) or KU-0060648 and etoposide phosphate (▼ once daily KU-0060648 dosing, Δ twice daily KU-0060648 dosing).

Europe PMC Funders Author Manuscripts

Table 1

Sensitisation of human colon and breast cancer cell lines to doxorubicin and etoposide by KU-0060648

Treatment	SW620	LoVo	MCF7	T47D	MDA-MB-231
1 μM Etop.	4.7±2.0	3.5±0.5	23±9	24±4	16±10
Etop. + KU	0.05±0.02	0.6±0.2	3.1±1.9	6.0±1.7	4.1±1.5
Fold enhancement*	**105±16**	**6.6±2.6**	**8.9±4.9**	**4.1±0.6**	**3.6±1.2**
100 nM Dox.	0.06±0.06	1.4±0.9	42.3±13	9.0±3.2	1.8±0.6
100 nM Dox. + KU	0.004±0.002	0.03±0.03	0.41±0.08	0.19±0.12	0.47±0.14
Fold enhancement*	**12±8**	**73±29**	**107±43**	**58±35**	**3.8±0.6**

Cells were exposed to etoposide (Etop), doxorubicin (Dox) and/or KU-0060648 (1 μM) for 16 hr prior to seeding for colony formation.

$$\text{fold enhancement} \quad \frac{\text{survival cytotoxic alone}}{\text{survival cytotoxic} + 1\mu\text{M KU} - 0060648} \quad (1)$$

Mol Cancer Ther. Author manuscript; available in PMC 2013 February 01.

Europe PMC Funders Author Manuscripts

Table 2

Plasma PK following different routes of administration and tumour and plasma concentration following a 12.5 mg/kg dose i.v. in mice bearing s.c. MCF7 or SW620 human xenografts

KU-0060648 Plasma PK after different routes of administration				**[KU-0060648] μM in tumour and plasma in tumour-bearing mice after 12.5 mg/kg i.p.**			
Dose and route	**10 mg/kg i.v**	**10 mg/kg p.o**	**10 mg/kg i.p**	**MCF7**		**SW620**	
C_{max} (μg/ml)	1.9	0.9	0.9	**Plasma**	**Tumour**	**Plasma**	**Tumour**
T_{max} (min)	5	120	30				
AUC_{inf}(μg/ml*min)	242	287	189				
$T_{1/2}$ (min)	102	142	106				
CL (ml/kg/min)	41	35	54				
Bioavailability (%)		119	78				
60 min				1.0±0.6	7.0±3.8	2.1±0.5	4.9±0.3
120 min				0.8±0.5	4.7±1.1	0.7±0.2	2.9±0.4

Plasma PK data are calculated from mean values obtained at 5,10,15, 30, 60, 90, 120, 180, 240 and 360 min after administration from 3 mice per time point. C_{max} = concentration maximum, T_{max} = time of C_{max}, AUC = area under the curve, $T_{½}$ = elimination half-life, CL=clearance.

Plasma and tumour [KU-0060648] at 60 and 120 min Data are the means ± SD of values obtained from 3 mice/time point.