

The tubulysin analogue KEMTUB10 induces apoptosis in breast cancer cells via p53, Bim and Bcl-2

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Abstract

Purpose Tubulysins are natural tetrapeptides that inhibit tubulin polymerisation. Tubulysins are very potent inhibitors of mammalian cancer cell growth, but restricted availability has limited their characterisation and development as anti-cancer compounds. KEMTUB10 was recently developed as a synthetic analogue of natural tubulysins.

Methods The cell cytotoxicity of KEMTUB10 was studied in cell lines that represent the main breast cancer subtypes. The KEMTUB10 pro-apoptotic mechanism of action was studied in MCF7 and MDAMB231 cells.

Results KEMTUB10 exerts a potent cytotoxic effect in cells representing the main breast cancer sub-types. KEMTUB10 blocks cells in the G2/M phase of the cell cycle and is a strong stimulator of apoptosis/cell death. KEMTUB10-induced apoptosis involves p53 and Bim, and to some extent Bcl-2 phosphorylation.

Conclusions KEMTUB10 is a promising new anti-mitotic that exerts a potent cytotoxic effect in breast cancer

cells, blocks cells in the G2/M phase of the cell cycle and stimulates apoptosis/cell death. KEMTUB10 has a distinct mode of action to taxol, appears to be sensitive to different molecular factors in cells and is likely subject to different mechanisms of acquired resistance. KEMTUB10 has the potential to be an important addition to the anti-cancer therapeutic armoury.

Keywords Breast cancer · Tubulysin · Anti-mitotic · Apoptosis · p53 · Bcl-2

Introduction

The most common type of tumour in women is breast cancer, with more than a million new cases diagnosed annually (Jemal et al. 2011). There is ever-increasing evidence that breast cancer is composed of several distinct, but related, subtypes. Molecular profiling has identified at least five different subtypes (Sørlie et al. 2001; Dawson et al. 2013); luminal A, luminal B, HER2 overexpressing, basal-like and normal-like. Each subtype has a unique gene expression pattern (Sørlie et al. 2001) distinct prognosis (Brenton et al. 2005) and displays differential sensitivity to a range of anti-cancer drugs (Brenton et al. 2005). Most breast cancers are luminal (60–65 %) and typically express wild-type p53 protein. The other subtypes usually contain mutant or null p53. Approximately 20 % of breast tumours overexpress HER2, with the basal-like and normal-like subtypes accounting for around 10 and 5 %, respectively.

Paclitaxel (taxol) and docetaxel are widely used taxanes that are commonly used to treat breast cancer. Taxane-based neoadjuvant chemotherapy can be effective in HER2 overexpressing and basal-like tumours, producing higher pathological complete response rates than in luminal

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tumours (reviewed in Brenton et al. 2005). Taxanes bind to β -tubulin, stabilising the microtubules and preventing normal formation of mitotic spindles (Jordan et al. 1996). This leads to chronic activation of the spindle assembly checkpoint, mitotic arrest (Musacchio and Salmon 2007) and subsequently to cell death. The mechanism of cell death is likely cell type dependent; some studies have observed a classical apoptotic response (Allan and Clarke 2007; Panvichian et al. 1998), whereas others have detected caspase-independent death (Niikura et al. 2007). Resistance to taxane treatment has been attributed to various factors (reviewed in Murray et al. 2012), including overexpression of the anti-apoptotic protein Bcl-2, the p-glycoprotein membrane transporter and the HER2 receptor. p53 status may also influence the sensitivity of cells to taxanes. However, this hypothesis is controversial, as research studies have observed that the cellular taxol IC_{50} value can be

increased (Wu and El-Deiry 1996), decreased (Perego et al. 1998) or not affected (Vasey et al. 1996) in cells lacking active p53.

Tubulysins are natural tetrapeptides produced by myxobacteria that bind to tubulin near the vinca alkaloid binding site. They inhibit tubulin polymerisation and are very potent inhibitors of mammalian cancer cell growth, with IC_{50} values ranging from 0.01 to 10 nM (Sasse et al. 2000). Restricted availability of tubulysins has limited their characterisation and development as anti-cancer compounds. Synthetic and medicinal chemists have recently addressed this issue by developing a synthetic range of tubulysin analogues. Synthetic and medicinal chemists have recently addressed this issue by developing a synthetic range of tubulysin analogues. KEMTUB10 is a synthetic tubulysin analogue where the natural substituent on the central N-tubuvalline fragment has been replaced with a more chemically stable benzyl group, and the C-terminal tubuphenylalanine incorporates a fluorine atom in para-position on the phenyl ring

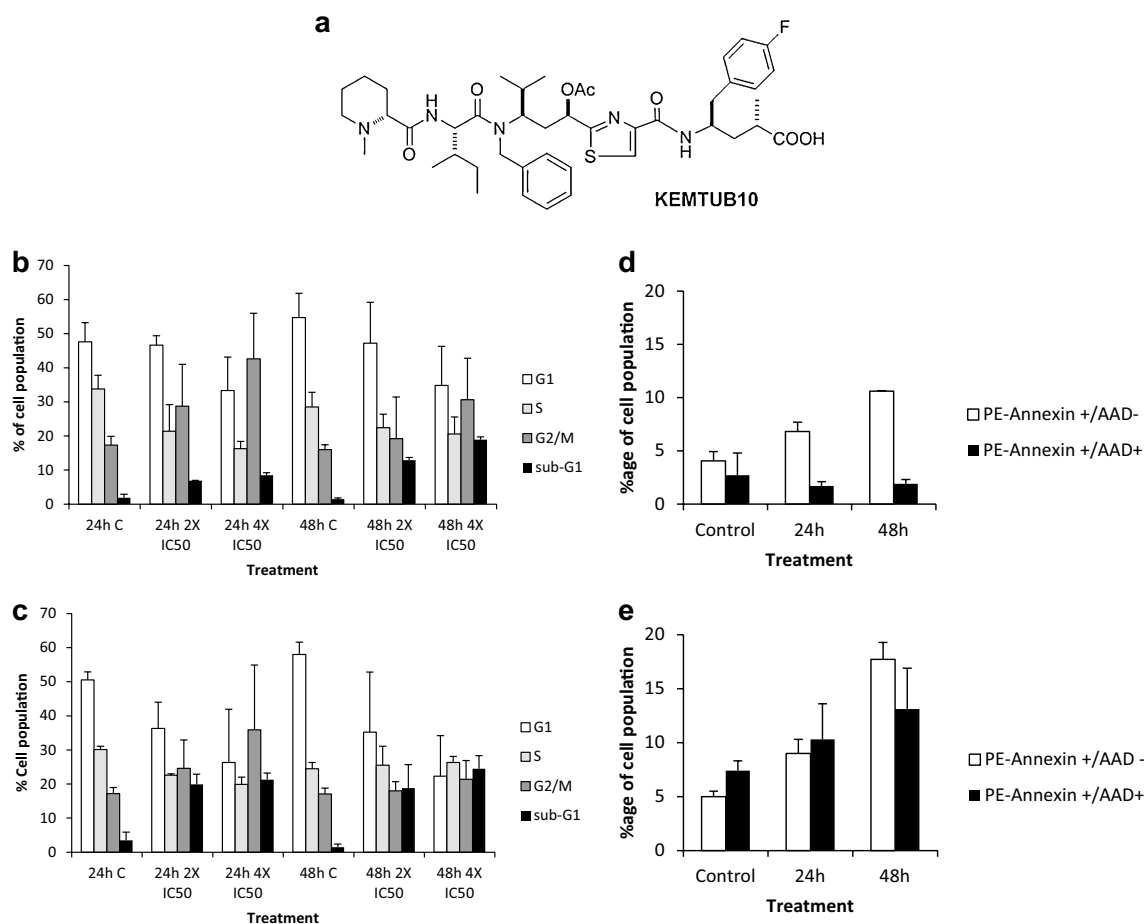


Fig. 1 KEMTUB10 increases the G2/M and sub-G1 cell populations in breast cancer cells. **a** Chemical structure of KEMTUB10. MCF7 (**b**) and MDA231 (**c**) cells were treated with DMSO, 2 \times or 4 \times IC_{50} KEMTUB10 for 24 or 48 h before harvesting. The DNA content of the cells was analysed by flow cytometry on a FACSCalibur flow cytometer (Becton–Dickinson) after propidium iodide stain-

ing. MCF7 (**d**) and MDA231 (**e**) cells were treated with DMSO or 2 \times IC_{50} KEMTUB10 for 24 or 48 h. Apoptotic (PE-Annexin V+/7-AAD $-$) and dead (PE-Annexin V+/7-AAD $+$) cells were detected using a PE-Annexin V/7-AAD apoptosis detection kit; 1 \times IC_{50} values for MCF7 and MDA231 cells were 30.1 and 68 pM, respectively. Results are the average \pm SD from three independent experiments

(Fig. 1a). KEMTUB10 and natural tubulysins both show potent cytotoxicity towards different tumour cell lines, but KEMTUB10 has the advantage of much increased availability. It can be synthesised in one synthetic cycle according to the efficient procedure developed by KemoTech Srl (Matteo Zanda, Monica Sani, Paolo Lazzari: “Pharmaceutical compounds” patent number US 8,580,820); US (US 2011/200581).

Initial studies have provided preliminary evidence that tubulysins can induce apoptosis/cell death in human cancer cells (Kaur et al. 2006; Khalil et al. 2006; Herrmann et al. 2012), but little is known about the underlying molecular mechanism, whether apoptosis/cell death makes a significant contribution to tubulysin cytotoxicity or the molecular factors that define tubulysin sensitivity/resistance. A better understanding of these factors is required to optimise the development and clinical application of this new drug class. In this study, we evaluated the cytotoxicity of KEMTUB10 in cancer cell lines that represent different breast cancer subtypes. Flow cytometry analysis was used to determine the effect of KEMTUB10 on cell cycle profiles. Finally, Western blot analysis was used in combination with molecular biological approaches and a pharmacological inhibitor to identify key proteins involved in KEMTUB10-induced apoptosis.

Methods

Cell lines, cell culture and reagents

Experiments were performed in MCF7, MDAMB231 (MDA231), SkBr3 and MDAMB453 (MDA453) breast cancer cells. MCF7 represents the luminal subtype, which characteristically express oestrogen and progesterone receptors (Sørlie et al. 2001). MDA231 represents the basal-like subtype, which typically does not contain oestrogen, progesterone or HER2 receptors and generally has a poorer prognosis than luminal tumours. SkBr3 and MDA453 both overexpress HER2. Some experiments also involved MCF7 DD cells, an MCF7 variant that stably expresses a dominant-negative p53 construct to abrogate p53 function (Shaulian et al. 1992; Smith et al. 2006).

MCF7, MDA231, SkBr3 and MDA453 cells were purchased from ATCC and confirmed as authentic and contamination-free. MCF7 DD cells were a kind gift from Alastair Thompson (university of Dundee). Cell cultures were maintained in RPMI containing 10 % (v/v) foetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin and grown at 37 °C in a humidified atmosphere containing 5 % CO₂. MCF7 DD cultures also contained 100 µg/ml hygromycin B (Fisher Scientific) to prevent loss of the dominant-negative p53 construct during propagation; p53

expression was regularly monitored by Western blotting. All reagents were purchased from Sigma-Aldrich, unless stated otherwise.

Cell cytotoxicity assays

All cell lines were seeded in 96-well plates at 5,000 cells/well, except SkBr3, which was seeded at 3,500 cells/well and then left to settle overnight. Stock solutions of KEMTUB10 (KemoTech Srl, Italy, www.kemotech.it), taxol, docetaxel and doxorubicin were prepared in DMSO. Cells were treated with a range of drug concentrations for 72 h in triplicate wells. The number of viable cells in each well was estimated by incubating cells in media containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation reagent for 1–2 h. Medium was aspirated; the formazan product was solubilised in DMSO (Smith et al. 2013) and measured at 540 nM in a microplate reader. GraphPad Prism was used to calculate IC₅₀ values.

Western blot analysis

MCF7, MCF7 DD and MDA231 cells were seeded at $0.35 \times 10^6/60$ mm plate and left to settle overnight. Cells were incubated with DMSO, KEMTUB10, taxol or docetaxel at the specified concentration for the time indicated. The protein concentration in the resulting lysates was determined by BCA assay. Blotting was done essential as described previously (Green et al. 2010), using the following antibodies: β-Actin (Sigma); p53, Mcl-1, Bcl-x l/s (all from Insight Biotechnology); Bcl-2, pser70 Bcl-20, Bid, Bim, cPARP (all from Cell signalling); and Noxa (Calbiochem). Images were captured by a Fluor-S Multiimager (Bio-Rad) using Quantity 1 software. Densitometries of target proteins were determined using ImageJ (National Institutes of Health).

Flow cytometry cell analysis

Cells were seeded and treated as described for Western blot experiments. KEMTUB10-treated cells were harvested, and the cell cycle profile of KEMTUB10-treated cells was analysed by propidium iodide staining of ethanol-fixed cells, as described previously (Fleming et al. 2008). A PE-Annexin V/7-Amino-Actinomycin (7-AAD) apoptosis detection kit (BD Pharmingen) was used to distinguish between apoptotic (PE-Annexin V+/AAD−) and dead (PE-Annexin V+/AAD+) cells, as recommended by the manufacturer.

Trypan blue exclusion assay to detect apoptotic/dead cells

Cells were seeded as described for Western blot experiments and then treated with the indicated concentrations

Table 1 KEMTUB10, taxol, docetaxel and doxorubicin IC₅₀ values in select breast cancer cell lines in a 72-h cell cytotoxicity assay. MCF7 (p53 wt) and MCF7 DD (dominant-negative P53) are paired cell lines that differ only in p53 status

	MCF7	MCF7 DD	MDA231	MDA453	SkBr3
KEMTUB10 (pM)	30.1 ± 13.2	68.6 ± 30.1	68 ± 18	16.3 ± 5.6	12.2 ± 6
Taxol (nM)	1.7 ± 0.4	1.5 ± 1.1	1.7 ± 0.8	1.9 ± 0.6	1.5 ± 0.2
Docetaxel (nM)	2.3 ± 0.4	1.21 ± 0.5	Not tested	Not tested	Not tested
Doxorubicin (nM)	51.7 ± 16.5	149.3 ± 18.3	Not tested	Not tested	Not tested

Result is the average ± SD of at least three independent experiments performed in triplicate. Statistical significance of IC₅₀ differences between MCF7 and MCF7 DD cells: KEMTUB10 $P < 0.05$; Taxol $P > 0.05$; Docetaxel < 0.05 ; Doxorubicin $P < 0.01$

of KEMTUB10 for 48 h. Adherent cells in each plate were trypsinised and combined with floating cells collected by centrifugation (1,000g × 4 min) from the culture medium. Cells were incubated with trypan blue for 5 min, and then the proportion of blue (dead/apoptotic) and transparent (live) cells was counted in a haemocytometer (Blagosklonny et al. 2002).

siRNA interference of Bim

MCF7 cells were seeded at 1×10^5 cells/well in 6-well plates in antibiotic-free medium and left to settle overnight. Next day, cells were transfected with 10 µl of 10 µM of control (non-specific) or Bim siRNA (both from Insight Biotechnology, UK) using oligofectamine (Fisher Scientific). Cells were then left for 24 h prior to treatment with DMSO or KEMTUB10 for 40 h. Bim expression and cPARP induction were assessed by Western blot as described above.

Results

HER2 and p53 expression was checked in the model cell lines by Western blotting (data not shown). The results confirm that our breast cancer cell line clones have the expected expression profiles. The SKBr3 and MDA453 cells expressed much higher HER2 protein levels than MCF7, MCF7 DD or MDA231. MCF7 cells contained modest p53 levels, consistent with a cell line expressing wt p53. MCF7 DD, SkBr3 and MDA231 cells expressed high p53 protein levels, consistent with cells expressing mutant/inactive p53. MDA453 lysates did not contain any detectable p53 protein, in agreement with published data (Zhou et al. 2012).

KEMTUB10 induced a dose-dependent increase in cell cytotoxicity in MCF7, MDA231, MDA453 and SkBr3 cells (data not shown). Picomolar IC₅₀ values were calculated in these four diverse cell lines (Table 1), ranging from 12.2 pM in SkBr3 to 68 pM in MDA231. Under identical conditions, taxol generated IC₅₀ values of 1–2 nM in MCF7, MDA231, MDA453 and SkBr3 cells (Table 1).

Flow cytometry was used to assess the effect of KEMTUB10 treatment on the cell cycle profile of MCF7 and

MDA231 cells. In MCF7 cells, KEMTUB10 caused dose-dependent decreases in the G1 and S populations after 24 and 48 h (Fig. 1b). KEMTUB10 increased the G2/M cell population by around 25 % after 24 h, which decreased thereafter. KEMTUB10 also induced a significant increase in the sub-G1 cell population after 24- and 48-h treatment; approximately 19 % of cells were sub-G1 after 48-h treatment with $4 \times$ IC₅₀. In MDA231 cells (Fig. 1c), there were very similar decreases in the G1 and S-phase populations and increases in the G2/M and sub-G1 cell populations, although there was little increase in the sub-G1 population after 24-h treatment. In both cell lines, KEMTUB10 treatment induced time-dependent increases in apoptotic cells (Fig. 1d, e). In MCF7 cells, cell death was entirely through the apoptosis pathway, whereas MDA231 cells die via both apoptotic and non-apoptotic mechanisms.

Lysates were analysed by Western blotting to identify molecular changes that could account for the observed KEMTUB10-induced apoptosis. In MCF7 cells, KEMTUB10 induced a robust time-dependent increase in cleaved PARP (cPARP) (Fig. 2a), a well-established biomarker of apoptosis. A more modest increase in cPARP was observed in MDA231 cells (Fig. 2b). These data confirm that KEMTUB10 induces apoptosis in breast cancer cells. Further investigation identified noticeable changes in several apoptosis-related proteins in MCF7 cells prior to the appearance of cPARP, suggesting that they may contribute to the increased apoptosis. These changes included induction of p53 and the p53-regulated pro-apoptotic protein Noxa, upregulation of the short (S) and long (L) versions of the pro-apoptotic protein Bim, and phosphorylation of the anti-apoptotic protein Bcl-2 at serine70. In contrast, there were no apparent changes in the levels of Mcl-1, Bcl-2, Bcl-x l/s or Bid prior to cPARP induction, suggesting that they do not play a significant role in KEMTUB10-induced apoptosis. Bim upregulation and Bcl-2 phosphorylation were also observed in MDA231 cells, providing confirmation that these proteins are regulated by KEMTUB10.

Further investigation of the KEMTUB10 apoptotic mechanism was performed in MCF7 cells, since cell death is primarily mediated via apoptosis in that cell line. Initial experiments examined the potential role of Bcl-2

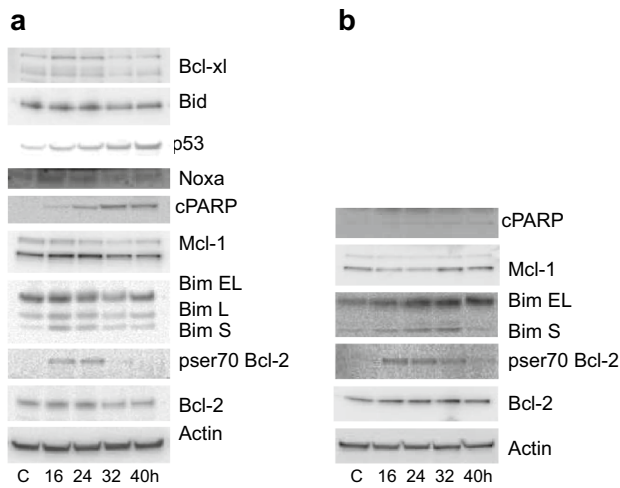


Fig. 2 KEMTUB10 selectively modulates apoptotic proteins in breast cancer cells. MCF7 (a) and MDA231 cells (b) were treated with DMSO (c) or $2 \times IC_{50}$ KEMTUB10 for 16, 24, 32 or 40 h prior to harvesting. Cell lysates (10–20 μ g) were resolved on 4–12 or 12 % acrylamide bis–tris gels, then transferred to PVDF membranes and probed with the antibodies shown. cPARP = cleaved PARP. EL, L and S represent the extra-long, long and short versions of Bim. Result is representative of two independent experiments

phosphorylation. Phosphorylation at serine 70 and serine 87 is associated with loss of the anti-apoptotic function of Bcl-2 (Haldar et al. 1995), and taxane-induced phosphorylation of these residues by c-Jun N-terminal kinase (JNK) makes an important contribution to taxol-induced apoptosis (Shajahan et al. 2012). MCF7 cells were treated with equivalent ($2 \times IC_{50}$) concentrations of KEMTUB10, taxol or docetaxel to compare the ability of these agents to stimulate Bcl-2 phosphorylation and apoptosis in that cell line. The anti-mitotics induced similar increases in cPARP and Bcl-2 phosphorylation (Fig. 3a). These data indicate that increased apoptosis is likely to play equally important roles in the anti-cancer activity of these agents. Cells were treated with the well-established JNK inhibitor SP600125 (Shajahan et al. 2012) to investigate whether Bcl-2 phosphorylation is involved in KEMTUB10-induced apoptosis. SP600125 caused similar decreases in taxol- and KEMTUB10-induced Bcl-2 phosphorylation (Fig. 3b, c). SP600125 reduced taxol-induced cPARP induction by approximately 65 %, but only decreased KEMTUB10-induced apoptosis by approximately 25 % (Fig. 3b, d). This is the first evidence that tubulysin-induced apoptosis involves Bcl-2 phosphorylation. However, the relatively modest ability of SP600125 to inhibit KEMTUB10-induced apoptosis suggests that other factors are also involved.

An siRNA that targets Bim was used to specifically downregulate Bim and determine whether it may

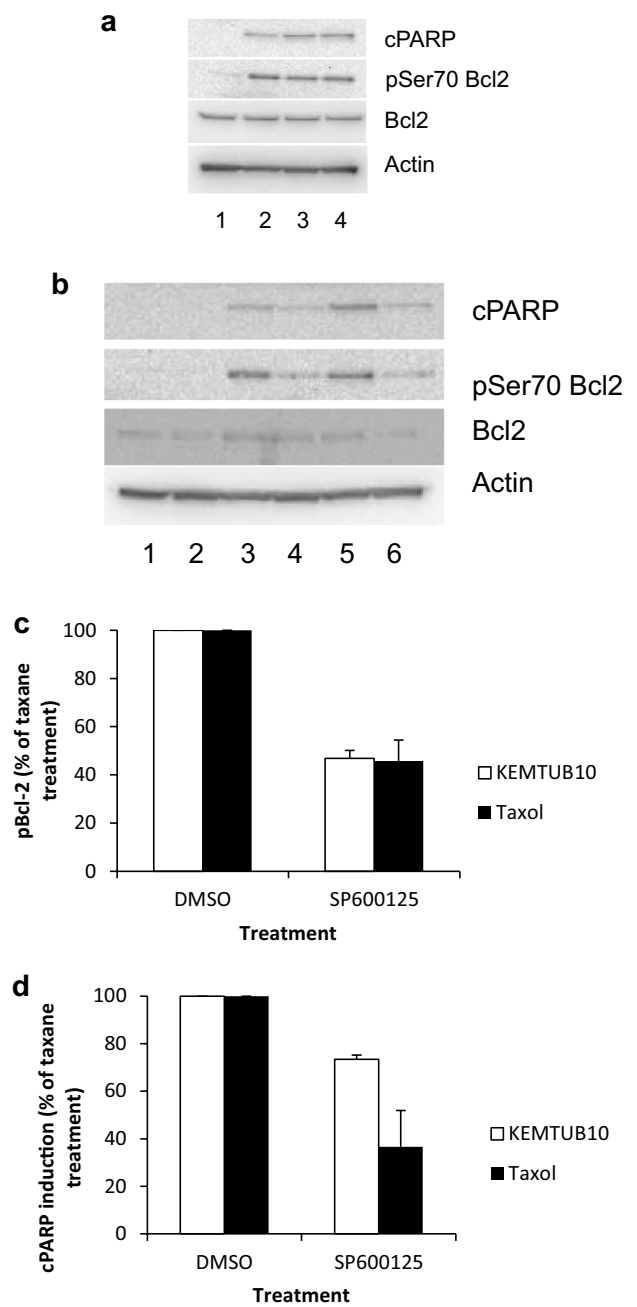


Fig. 3 Bcl-2 phosphorylation contributes to KEMTUB10-induced apoptosis in MCF7 cells. **a** MCF7 cells were treated with DMSO (1), $2 \times IC_{50}$ KEMTUB10 (2), $2 \times IC_{50}$ taxol (3) or $2 \times IC_{50}$ docetaxel (4) for 48 h. **b** MCF7 cells were incubated with DMSO (1), 10 μ M SP600125 (2), $2 \times IC_{50}$ KEMTUB10 (3), $2 \times IC_{50}$ KEMTUB10 + 10 μ M SP600125 (4), $2 \times IC_{50}$ taxol (5) or $2 \times IC_{50}$ taxol + 10 μ M SP600125 (6) for 48 h. The resulting lysates (a, b) were probed for cPARP, pser70 Bcl-2, Bcl-2 and actin. The average band intensity of pSer70 Bcl-2 (c) and cleaved PARP (d) bands was measured by densitometry. Result is representative (a, b) or average \pm SD (c, d) of at least three independent experiments; $1 \times IC_{50}$ values for KEMTUB10, taxol and docetaxel were 30 pM, 1.7 nM and 2.3 nM, respectively. *T* test values SP600125 inhibition of Bcl-2 phosphorylation, Taxol vs KEMTUB10 treatment $P > 0.05$; cPARP induction, Taxol versus KEMTUB10 treatment $P < 0.05$

contribute to KEMTUB10-induced apoptosis. Preliminary experiments established that the Bim protein bands were decreased by approximately 60 % 24 h after transfection of MCF7 cells, and by 75 % after 48 h (not shown). Bim siRNA treatment decreased Bim levels to a similar extent in the presence or absence of KEMTUB10 treatment (Fig. 4a, b). KEMTUB10-induced apoptosis was decreased approximately 67 % by Bim siRNA treatment (Fig. 4a, c). These data confirm that Bim plays an important role in tubulysin-induced apoptosis and that it makes a more significant contribution than Bcl-2 inhibition.

A possible role for p53 in the KEMTUB10 mode of action was investigated using MCF7 wt and DD cells, paired cell lines that differ only in their p53 status. The KEMTUB10 IC_{50} curve in MCF7 DD cells was shifted to the right compared to MCF7 wt cells (Fig. 5a), corresponding to a doubling in the IC_{50} value (Table 1). The doxorubicin IC_{50} value was also significantly higher in MCF7 DD than wt cells (Table 1), consistent with literature demonstrating that doxorubicin-induced apoptosis is greater in p53 wt than p53 mutant cells (Vasey et al. 1996) and in cells that overexpress p53 (Blagosklonny et al. 1998). In contrast, taxol IC_{50} values were similar in the paired cell lines. Subsequently, the effect of p53 status on KEMTUB10-induced apoptosis was tested. In MCF7 wt cells 2 \times , 4 \times and 10 \times IC_{50} KEMTUB10 induced cPARP production (Fig. 5b, c). However, in MCF7 DD cells, cPARP was only detected after treatment with 10 \times IC_{50} KEMTUB10. Densitometry analysis indicated that 10 \times IC_{50} KEMTUB10 produced approximately 75 % less cPARP in DD than wt cells. These data provide strong evidence that the p53 status of cells influences KEMTUB10-induced apoptosis. This concept was confirmed using a trypan blue exclusion assay to detect apoptotic/dead cells. Compared to control cells, 2 \times IC_{50} KEMTUB10 caused 10.2 and 2.7 % increases in apoptotic/dead MCF7 wt and DD cells, respectively (Fig. 5d). The equivalent values for 4 \times IC_{50} KEMTUB10 were 15.2 and 6 %. These data demonstrate that KEMTUB10-induced cell death/apoptosis is significantly lower in MCF7 DD than wt cells.

Discussion

Anti-mitotic drugs are an important constituent of several well-established therapies for the treatment or management of breast cancer (Brenton et al. 2005). Taxanes are more efficacious than many other anti-cancer drugs in certain breast cancer sub-types, but there are still some patients

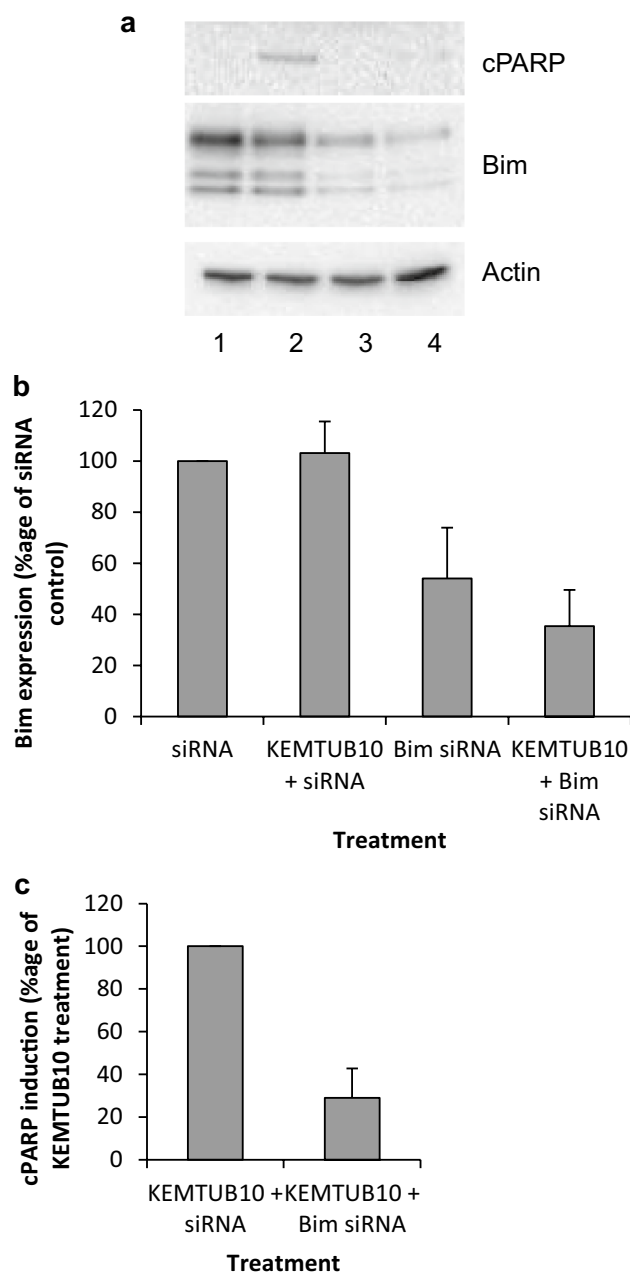


Fig. 4 Bim plays an important role in KEMTUB10-induced apoptosis in MCF7 cells. **a** MCF7 cells were transfected with control siRNA (lanes 1 and 2) or Bim siRNA (lanes 3 and 4) prior to treatment with DMSO (lanes 1 and 3) or 2 \times IC_{50} KEMTUB10 (lanes 2 and 4) for 40 h. The resulting lysates were probed for Bim, cPARP and actin. The average band intensity of Bim (**b**) and cleaved PARP (**c**) bands was measured by densitometry. Result is representative (**a**) or average \pm SD (**b**, **c**) of at least three independent experiments; 1 \times IC_{50} values for KEMTUB10 was 30 pM. *T* test values Bim down-regulation, Bim siRNA versus Bim siRNA + KEMTUB10 $P > 0.05$; cPARP induction, KEMTUB10 + control siRNA versus KEMTUB10 + Bim siRNA $P < 0.01$

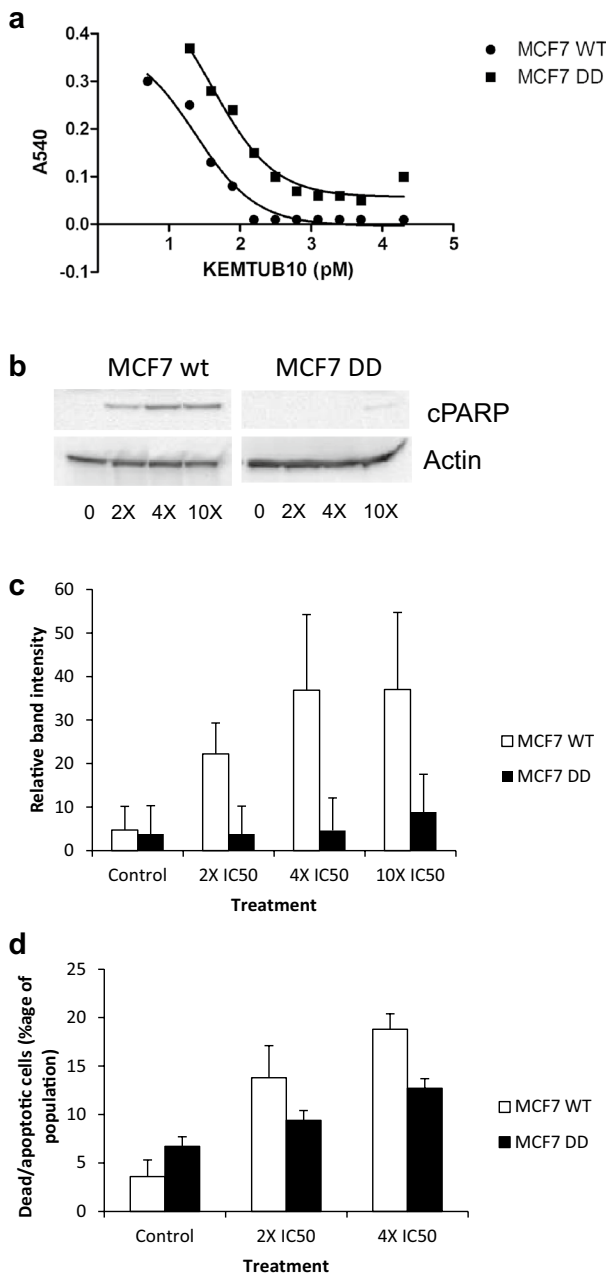


Fig. 5 P53 plays a key role in KEMTUB10-induced apoptosis in MCF7 cells. **a** KEMTUB10 IC₅₀ curves in MCF7 and MCF7 DD breast cancer cells. X-axis is a log scale. **b** MCF7 wt and DD cells were incubated with 2×, 4× or 10× IC₅₀ KEMTUB10 for 48 h, and the resulting lysates probed for cPARP and actin by Western blotting. **c** The average band intensity of the cleaved PARP bands was measured by densitometry. **(d)** The effect of KEMTUB10-treatment on apoptosis/cell death was determined using a trypan blue cell exclusion assay. Result is representative (**a**, **b**) or average ± SD (**c**, **d**) of at least three independent experiments. *T* test values cPARP induction MCF7 versus MCF7 DD at 2×, 4× and 10× IC₅₀ all *P* < 0.05; trypan blue exclusion MCF7 versus MCF7 DD at 2× and 4× IC₅₀ both *P* < 0.05

who do not respond to treatment, and development of taxane resistance is a growing problem. Accordingly, there is significant interest in the development of new anti-mitotics with higher potencies and distinct modes of action, as they may be efficacious in a higher proportion of patients, in different breast cancer sub-types and/or in tumours that develop taxane resistance.

Tubulysins are a potent class of anti-mitotics that have the potential to be efficacious anti-cancer drugs. KEMTUB10, a new synthetic analogue of tubulysin, produced picomolar IC₅₀ values in cell lines representing the main breast cancer subtypes (Table 1). Tubulysins generated IC₅₀ values of a similar magnitude, ranging from 10 pM to 10 nM, in cell lines derived from various tumours (Khalil et al. 2006). In contrast, pretubulysin was approximately 10- to 100-fold less active in tumour cell lines (Herrmann et al. 2012). SkBr3 and MDA453 cells appeared to be particularly sensitive to KEMTUB10 (Table 1), providing preliminary evidence that this anti-mitotic may have high efficacy in HER2 overexpressing cells. This is an intriguing observation that will be investigated further in future studies. In contrast to KEMTUB10, taxol IC₅₀ results did not vary significantly between sub-types (Table 1). This suggests that KEMTUB10 and taxol are sensitive to different molecular factors in cells. KEMTUB10 is approximately 20–120 times more potent than taxol in cultured breast cancer cells. This could potentially translate into higher efficacy against cancer cells or prescribing a lower drug dose to patients.

In breast cancer cells, 2×–4× IC₅₀ KEMTUB10 increased the proportion of G2/M cells by up to 25 % (Fig. 1). Tubulysin A (Khalil et al. 2006; Herrmann et al. 2012) and pretubulysin (Herrmann et al. 2012) also cause large G2/M increases in cancer cells. These data confirm that tubulysins produce the G2/M cell cycle accumulation that is characteristic of tubulin-disrupting/anti-mitotic agents. In MCF7 and MDA231 cells, KEMTUB10 treatment also caused a significant (up to 19 %) increase in the sub-G1 cell population (Fig. 1), which is usually characteristic of cell death/apoptosis. Published studies involving tubulysin A (Khalil et al. 2006; Herrmann et al. 2012) or pretubulysin (Herrmann et al. 2012) have not reported significant increases in sub-G1 cell populations. However, this is likely a function of the cell lines studied, since tubulysins do induce apoptosis in other cancer cell lines (Kaur et al. 2006; Khalil et al. 2006; Herrmann et al. 2012).

Four independent data sets provide evidence that KEMTUB10 induces apoptosis/cell death in breast cancer cells: (1) KEMTUB10-induction of apoptotic cells with the annexin V/AAD assay (Fig. 1d, e), (2) time- and

dose-dependent increase in sub-G1 cells (Fig. 1b, c), (3) time-dependent (Fig. 2) and dose-dependent (Fig. 5b, c) increase in cPARP and (4) dose-dependent increase in dead/apoptotic cells observed in the trypan blue permeability assay (Fig. 5d). KEMTUB10 treatment caused similar increases in the sub-G1 cell population (Fig. 1b, c) and apoptotic/dead cells (Figs. 1d, e, 5d). These findings demonstrate that KEMTUB10 is a potent stimulator of cell death in breast cancer cells and that this is likely to make an important contribution to its anti-cancer effect. Indeed, when MCF7 cells were treated with equivalent ($2 \times IC_{50}$) concentrations of KEMTUB10, taxol or docetaxel, similar increases in cPARP were observed (Fig. 3a), indicating that increased apoptosis is likely to play equally important roles in the anti-cancer activity of these agents.

Interestingly, KEMTUB10 induced a significant increase in the sub-G1 cell population in MCF7 (Fig. 1b) and MDA231 (Fig. 1c) cells, but the mechanism of cell death appears to be subtly different in these cell lines. In MCF7 cells, KEMTUB10 appears to induce a classical apoptotic response (Allan and Clarke 2007; Panvichian et al. 1998), as demonstrated by a strong induction of PE-Annexin V+/7-AAD-cells (Fig. 1d) and cleaved PARP induction (Fig. 2a). In contrast, in MDA231, cell death was primarily via apoptosis (Fig. 1e), but did not involve induction of cleaved PARP (Fig. 2b). The most likely explanation is that MDA231 cells utilise a caspase-independent apoptotic mechanism, equivalent to that described in taxol-treated cells (Niikura et al. 2007). Anti-mitotic drugs are likely to use several different mechanisms to kill cancer cells (Gascoigne and Taylor 2009). The molecular factors that determine which pathway(s) predominate in a particular cell line are not yet clear.

This is the first paper to identify the key proteins involved in tubulysin-stimulated apoptosis. Western blotting indicated that KEMTUB10 stimulated upregulation of p53 and Bim, and phosphorylation of Bcl-2 prior to cPARP induction (Fig. 2), suggesting that these early events likely contribute towards the increased apoptosis. The importance of these factors was confirmed using a well-established c-Jun N-terminal kinase (JNK) inhibitor (Fig. 3), siRNA knockdown of Bim (Fig. 4) and paired MCF7 cell lines that express wt or mutant p53 (Fig. 5; Table 1). Activation of p53 signalling was confirmed by Noxa upregulation (Fig. 2), a pro-apoptotic protein of the Bcl-2 family that is transcriptionally regulated by p53. Indeed, p53 apparently plays a critical role in the KEMTUB10 mode of action, as p53 status influences the KEMTUB10 IC_{50} value (Fig. 5a; Table 1), and KEMTUB10-stimulation of both cPARP (Fig. 5b, c) and cell death/apoptosis (Fig. 5d). These data will help to provide a better understanding of the factors that determine tubulysin sensitivity/resistance and therefore

which breast cancer sub-types are more likely to respond to KEMTUB10 treatment.

Taxol IC_{50} values were not affected by p53 status (Table 1). The taxol data shown here are very similar to results generated previously in MCF7 DD cells (Hait and Yang 2006). This finding also agrees with the contrasting observations in the literature (Wu and El-Deiry 1996; Perego et al. 1998; Vasey et al. 1996) and the general consensus that p53 status does not significantly affect taxol sensitivity (Blagosklonny and Fojo 1999). Interestingly, experiments in MCF7 wt/DD cells suggest that vinblastine (Hait and Yang 2006) and KEMTUB10 (Fig. 5; Table 1) are similarly influenced by p53 status in this model. Vinca alkaloids and tubulysins both inhibit tubulin polymerisation, whereas taxol stabilises microtubules; one attractive hypothesis is that their disparate effects on tubulin polymerisation may account for their differing sensitivity to p53 status. This may, perhaps, be due to changes in expression of genes that are transcriptionally regulated by p53 (e.g. microtubule-associated protein 4) (Hait and Yang 2006). KEMTUB10-induced (Fig. 4) and taxol-induced apoptosis (Kutuk and Letai 2010) are both sensitive to Bim down-regulation, establishing that this broad specificity BH3-only protein regulates the anti-apoptotic effect of both anti-mitotics. On the other hand, abrogation of Bcl-2 phosphorylation had a larger inhibitory effect on taxol-induced than KEMTUB10-stimulated apoptosis (Fig. 3) (Shajahan et al. 2012). This may be because taxol-induced apoptosis is executed primarily via Bcl-2 inhibition and Bim induction, whereas KEMTUB10 also involves p53 (Fig. 5; Table 1). As Bcl-2 overexpression is a well-documented resistance mechanism for taxanes (reviewed in Murray et al. 2012), the lower reliance of KEMTUB10 on Bcl-2 phosphorylation may reduce its susceptibility to acquired Bcl-2 resistance.

The high potency of KEMTUB10 in breast cancer cells makes it an exciting prospect for further development as an anti-cancer agent. One attractive option would be to integrate KEMTUB10 into a protein conjugate for selective delivery to cancer cells. This could produce a highly selective anti-cancer drug that causes minimal toxicity in patients. KEMTUB10 has a distinct mode of action to taxol, so is sensitive to different molecular factors in cells and is therefore likely to be subject to different mechanisms of acquired resistance. KEMTUB10 has the potential to be an important addition to the anti-cancer therapeutic armoury.

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Conflict of interest The authors declare the following competing financial interest(s): P.L. is current employee of KemoTech s.r.l. Furthermore, M.S., P.L. and M.Z. are co-founders of KemoTech s.r.l. (www.kemotech.it). O.F.L. and I.N.F. have no conflicts of interest to disclose regarding this manuscript.

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