SPANDIDOS PUBLICATIONS

Differential enhancement of the anti-cancer effect of doxorubicin by Akt inhibitors on human breast cancer cells with differing genetic backgrounds

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Abstract. The phosphatidylinositol 3 OH-kinase (PI3K) pathway is a key intracellular signalling cascade in cellular survival. Our previous studies indicated that specific blockade of this enzyme led to sensitisation of human breast carcinoma cells to killing by doxorubicin through induction of both G2 arrest and apoptosis in some, but not all, breast cancer cells. In the present study, we report that inhibition of a downstream component of this pathway, Akt, is an effective means of enhancing doxorubicin killing in some breast cell types. Doxorubicin (Dox) and six Akt inhibitors were used individually or in combination on MDA-MB-231 (p53 mutant, ER^-), T47D (p53 mutant, ER^+), and MCF-7 (p53 wt, ER^+) human breast cancer cell lines. In MDA-MB-231 breast cancer cells, all six Akt inhibitors, which have differing mechanisms of action to inhibit Akt, synergised with the growth inhibitory effects of doxorubicin. Two Akt inhibitors also enhanced the effect of Dox in T47D cells but the other inhibitors induced additive effects in these cells. None of the inhibitors used elicited enhanced effects in MCF-7 cells. These results support the notion that combination therapies of doxorubicin (and possibly other chemotherapeutics) with inhibitors of elements of the PI3K pathway are a realistic possibility for future breast cancer therapy, which could lead to reduced side-effects, but that this could be dependent on the genetic background of each breast cancer.

Introduction

We have previously shown that MDA-MB-231 and T47D human breast cancer cell lines, but not MCF-7 cells, could be sensitised to the effect of doxorubicin by combining this chemotherapeutic with the PI3K inhibitor, LY294002 (1). The sensitisation appears to be through synergistic cell cycle G2 phase arrest and the enhanced induction of apoptosis. The possibility exists that similar or even greater enhancement might be achieved with the use of inhibitors of a downstream kinase. Akt (also known as protein kinase B, PKB) is a major substrate for PI3K and a primary mediator of PI3K-initiated signalling. Akt gene amplifications have been discovered in human gastric, ovarian, pancreatic and breast carcinomas (2-4). Akt3 mRNA overexpression and selective activation of the protein by growth factors have been observed in human hormone-independent breast and prostate cancer cell lines (5).

Furthermore, some studies have discovered an interaction between the activity of Akt and the response to cytotoxic agents. Ovarian cancer cells with overexpressed Akt are more resistant to paclitaxel than those with low Akt level (6). One study reported, that compared to hormone-sensitive prostate cancer cells, hormone-resistant cells have significantly increased amplification rate in the PI3K/Akt pathway (7). Also, overexpressed Akt is involved in the cisplatin chemoresistance of human uterine cancer cells (8).

Based on these findings, together with our reported synergy/additivity (1) in terms of blockade of PI3K in combination with doxorubicin, it was proposed that the direct inhibition of Akt activity might be able to sensitise further human breast cancer cells to the effect of doxorubicin, so possibly allowing a greater therapeutic efficacy of doxorubicin. In the present study, Akt inhibitor I, II, III, IV, V and VIII were used on human breast cancer cell lines, alone, or in combination with doxorubicin. The cell lines differed in two known parameters, namely p53 and estrogen receptor (ER) status: MDA-MB-231 (mutant p53, ER^-), T47D (mutant p53, ER^+) and MCF-7 (wild-type p53, ER^+).

Materials and methods

Cell culture. MDA-MB-231, MCF-7, T47D malignant human breast cell lines (ECACC, Porton Down, UK) were grown in continuous monolayer culture in sterile filter-top 75 cm^2 tissue culture flasks and were maintained in an incubator at 37˚C in a humidified atmosphere (5% CO_2/95% air) in growth medium DMEM (Dulbecco's Minimal Essential Medium, Gibco BRL, Paisley, UK) containing a final concentration of 2 mM

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L-glutamine (Biowhittaker Europe, Cambrex Bio Science, Verviers, Belgium). To this was added 10% (v/v) heat-inactivated FCS (Gibco BRL). Sub-culturing was performed twice weekly at a split ratio of 1:6 (MDA-MB-231 cell line), 1:4 (T47D cell line) and 1:5 (MCF-7 cell line). Cell lines were used within 10 passages, over which time there was no change in basal cell growth rate.

Cell incubations. After 24 h of incubation in growth medium, the medium was removed and cells were washed with phosphate-buffered saline (PBS) (Biowhittaker Europe, Cambrex Bio Science). Fresh growth medium was added to each well followed by addition of the drug according to the experiment. The Akt inhibitors (Calbiochem, Merck Biosciences Ltd., Nottingham, UK) were dissolved in DMSO and doxorubicin (Alexis Corp., Nottingham, UK) was dissolved in ultra-pure water. Unless stated otherwise, additions (vehicle, agonist) were pre-diluted in medium and control incubations were always treated with vehicle alone at exactly the same level as in the tests.

Assessment of cell proliferation. MDA-MB-231, T47D and MCF-7 were plated in 48-well plates at $1.0x10^4$/well, $2.0x10^4$/well and $1.5x10^4$/well respectively in the appropriate growth medium and then treated from 24 h onwards. After each appropriate incubation period, the medium was removed and the cells were washed in PBS. The cells were then harvested using 200 μl of trypsin-EDTA solution per well (Biowhittaker Europe, Cambrex Bio Science, Verviers, Belgium) and re-suspended individually. A Coulter Counter (Beckman Coulter UK Ltd., Bucks, UK) was used to assess the cell numbers. All results are reported as mean ± SEM (n=3). Coulter counting was selected on the basis of it being the most accurate determinant of the numbers of live cells. Each sample was counted in triplicate and the cell number/well calculated.

Data analysis. Student's t-test (two-tailed assuming unequal variance) and CalcuSyn software (Biosoft, Cambridge, UK) were used to determine the synergistic effect from combined treatment of two drugs, against the sum effect of these when used alone, with $P<0.05$ considered to be significant. All the data are presented as mean ± SEM (n=3) unless otherwise stated.

Furthermore, combination index (CI) was also calculated using the equation proposed by Chou and Talalay (9) with CI <1, 1 and >1 indicating synergism, an additive effect and antagonism, respectively.

Results

Dose-dependent growth inhibitory effects were observed in all three human breast cancer cell lines in response to 48 h treatment with Akt inhibitor I (5-50 μM), Akt inhibitor II (5-50 μM), Akt inhibitor III (2.5-40 μM). In addition, T47D cells and MDA-MB-231 cells showed dose responses to Akt inhibitor IV (0.05-1.0 μM), Akt inhibitor V (62.5-1000 nM), and Akt VIII (0.5-2.0 μM).

Akt inhibitor I. The effects of combined treatment with doxorubicin and Akt inhibitor I on MDA-MB-231, T47D and MCF-7 cell lines for 48 h are shown in Fig. 1. Synergistic inhibitory effects on the proliferation of MDA-MB-231 cells were observed from the combined treatment ($P<0.05$ and CI<1). For example, when used alone, 1 ng/ml of doxorubicin and 10 μM of Akt inhibitor I induced 3.33±0.71% and 16.82±1.24% of inhibitory effects compared to control, respectively. However, these two drugs worked in synergy when used in combination at the same doses to elicit 38.08±1.11% inhibition of proliferation, which could only have been obtained when using as high as 20 ng/ml of doxorubicin alone on this cell line. On T47D and MCF-7 cell lines, the growth inhibitory effects from combined treatments showed no significant difference compared to the sum effects of either drug alone (Fig. 1).

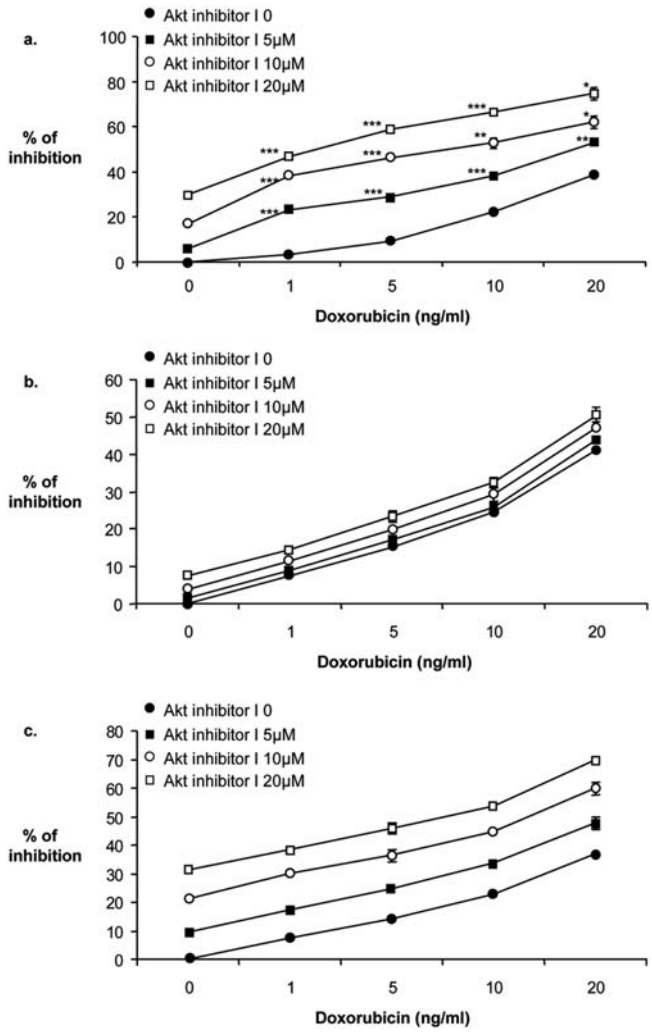

Figure 1. The effects of the combined treatment of doxorubicin and Akt inhibitor I after 48 h. Data were from three separate experiments. (a) In MDA-MB-231 cells the combination indicated synergy. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (as obtained from two-tailed Student's t-test assuming unequal variances). This was also confirmed by Combination Index (CI) <1. T47D (b) and MCF-7 (c) cells only showed additivity in response to the combined treatment of doxorubicin and Akt inhibitor I.

The results of identical experiments, to those depicted in Fig. 1, with doxorubicin combined with Akt inhibitors II, III, IV, V and VIII are shown in Figs. 2-6, respectively.

Akt inhibitor II. In Fig. 2, it is clear that synergistic inhibitory effects on proliferation were observed in both MDA-MB-231

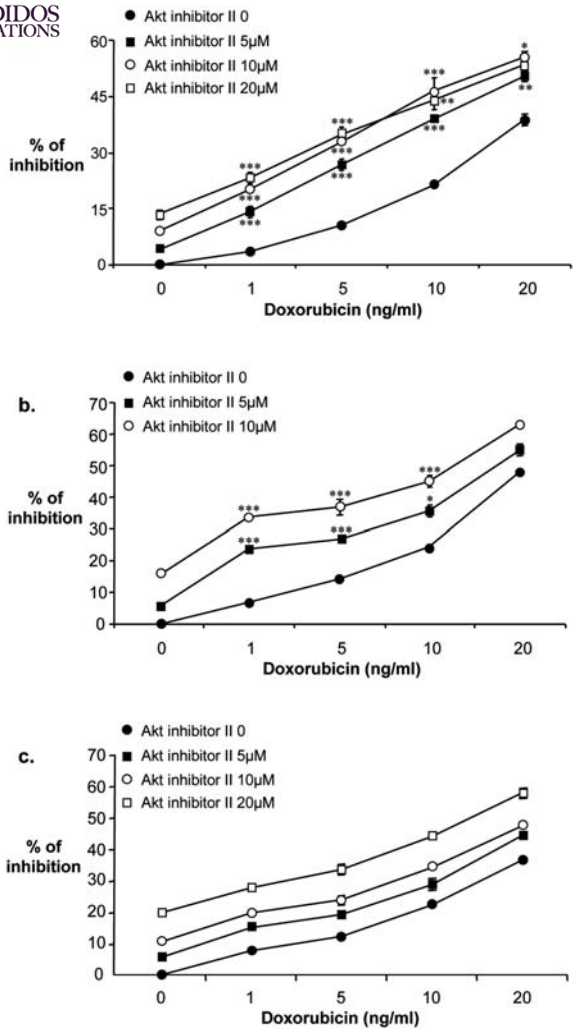

Figure 2. Growth inhibitory effects in response to the combined treatment of doxorubicin and Akt inhibitor II on (a) MDA-MB-231, (b) T47D cells and (c) MCF-7 cells. Error bars show SEM (n=3). In MDA-MB-231 and T47D cells, there was a synergistic response; *P<0.05; **P<0.01; ***P<0.001 (CI>1), whereas in MCF-7 cells only additivity was observed.

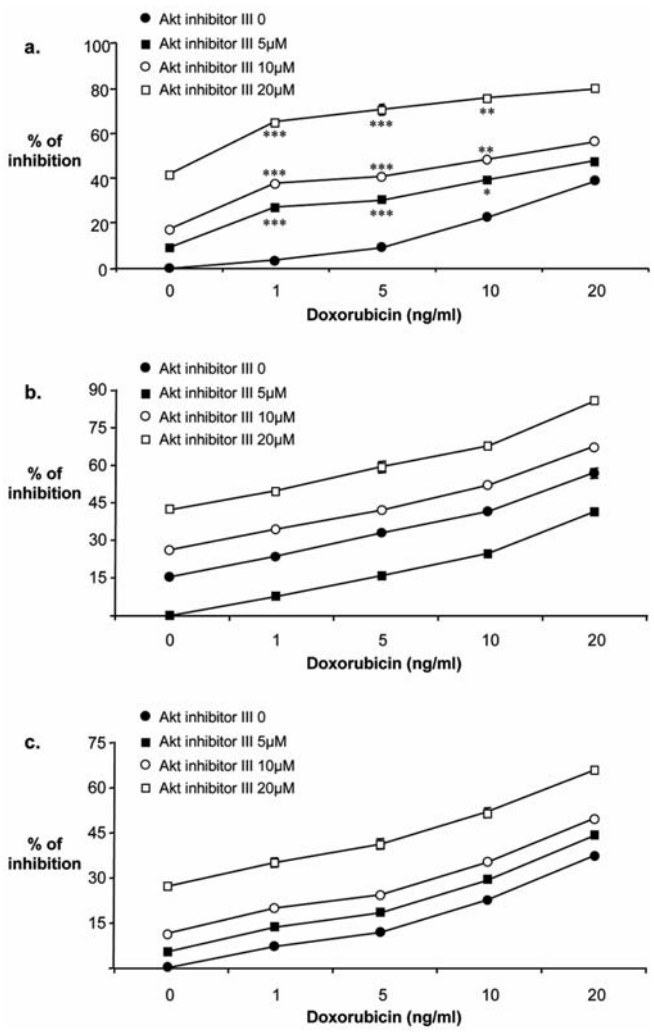

Figure 3. The growth inhibitory effects in response to the combined treatment of doxorubicin and Akt inhibitor III in (a) MDA-MB-231, (b) T47D and (c) MCF-7 cells. Data were from three separate experiments. Error bars show SEM (n=3). In MDA-MB-231 cells, *P<0.05; **P<0.01; ***P<0.001 (CI>1) indicating synergy.

and T47D cells (P<0.05, and CI<1). For example, when used alone on MDA-MB-231 cells, doxorubicin (5 ng/ml) and Akt inhibitor II (10 μM) induced 10.34±1.31% and 8.87±1.01% vs 32.72±1.59% inhibition in combination. Again only an additive inhibitory effect was observed from the combined treatment on MCF-7 cells.

Akt inhibitor III. It can be seen from Fig. 3 that synergistic inhibitory effects on the proliferation of MDA-MB-231 breast cancer cells were observed from the combined treatment (P<0.05 and CI<1). Used alone, 1 ng/ml of doxorubicin and 10 μM of Akt inhibitor III induced 3.63±0.81% and 17.06±1.12% inhibition respectively, compared to 37.96±1.08% inhibition of cell growth due to the combination. On the T47D and MCF-7 cell lines, only additive inhibitory effects were observed from the combined treatment.

Akt Inhibitor IV. The combined treatment with doxorubicin and Akt inhibitor IV on MDA-MB-231 and T47D cells each resulted in synergistic inhibitory effects (P<0.05 and CI<1) on proliferation (Fig. 4). For example, in MDA-MB-231 cells, the combination resulted in 50.82±2.32% inhibition in contrast to 21.39±1.64 and 13.4±1.35% for Akt inhibitor IV and doxorubicin, respectively.

Akt inhibitor V. Synergistic inhibitory effects on the proliferation of MDA-MB-231 breast cancer cells were observed from the combined treatment (Fig. 5). However, only an additive inhibitory effect was observed from the combined treatment on T47D cells. Akt inhibitor V (62.5 nM) alone and doxorubicin (1 ng/ml) alone had similar inhibitory effects on cell growth in these cells at 3.54±0.39 and 3.52±0.35%, respectively, but the combination resulted in 15.68±0.99% inhibition.

Akt inhibitor VIII. As shown in Fig. 6, synergy with doxorubicin and Akt inhibitor VIII was observed in MDA-MB-231 cells but not in T47D cells. In MDA-MB-231 cells doxorubicin (1 ng/ml) and Akt inhibitor VIII (0.05 μM) elicited 3.89±0.82 and 8.13±0.99% inhibition of proliferation,

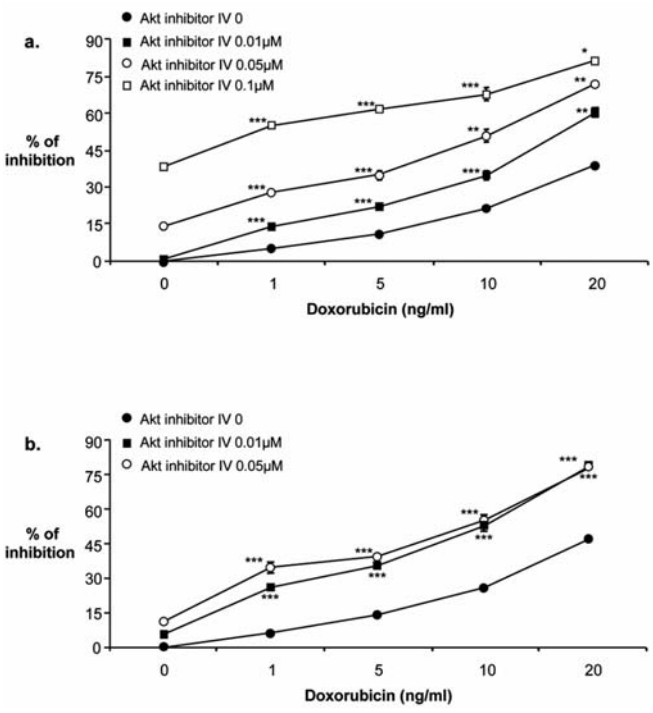

Figure 4. The growth inhibitory effects in response to the combined treatment of doxorubicin and Akt inhibitor IV in (a) MDA-MB-231 and (b) T47D. Error bars show SEM (n=3). *P<0.05; **P<0.01; ***P<0.001 (CI>1) indicating synergy.

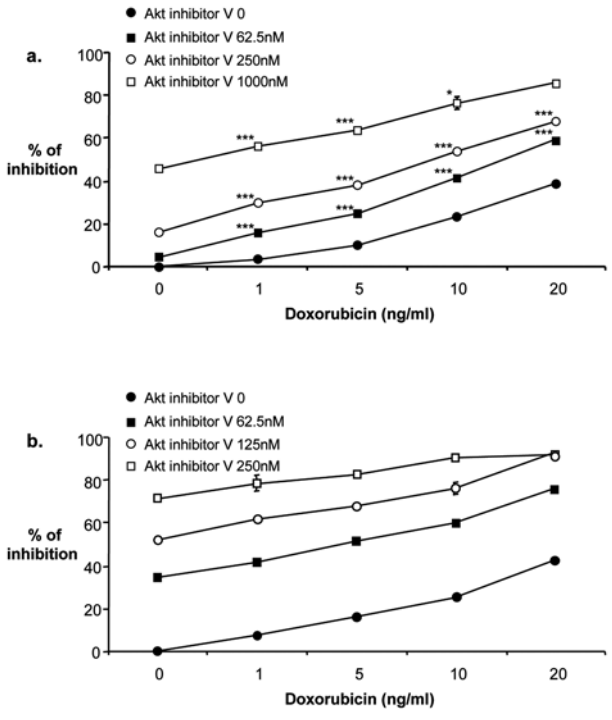

Figure 5. The growth inhibitory effects in response to the combined treatment of doxorubicin and Akt inhibitor V in (a) MDA-MB-231 and (b) T47D. Error bars show SEM (n=3). For MDA-MB-231 cells, *P<0.05; **P<0.01; ***P<0.001 (CI>1) indicating synergy.

respectively. Yet in combination an inhibition of 31.2±1.27% was achieved. On T47D cells, only an additive inhibitory effect was observed from the combined treatment.

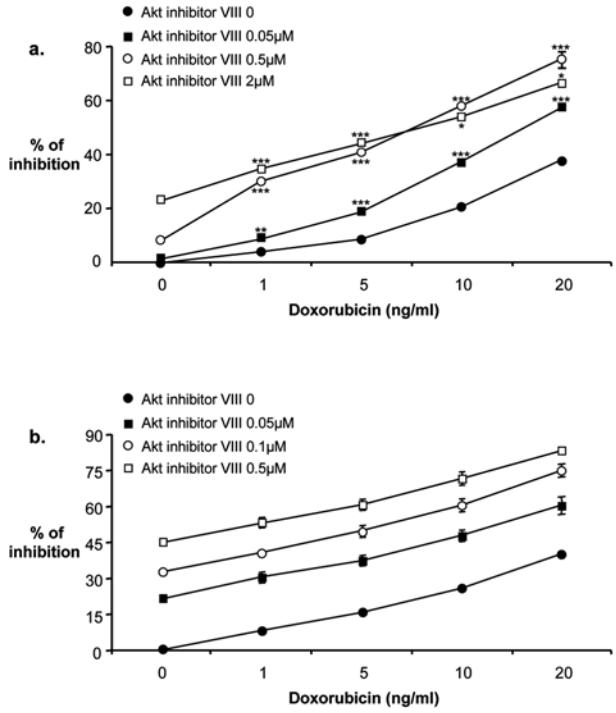

Figure 6. The growth inhibitory effects in response to the combined treatment of doxorubicin and Akt inhibitor IV in (a) MDA-MB-231 and (b) T47D. Error bars show SEM (n=3). For MDA-MB-231 cells, *P<0.05; **P<0.01; ***P<0.001 (CI>1) indicating synergy.

Discussion

The studies reported here focused on investigation of the growth inhibitory effect of Akt inhibitor I, II, III, IV, V and VIII alone or in combination with doxorubicin on human breast cancer cell lines to see whether these inhibitors could sensitise the breast cancer cells to the effect of doxorubicin. The aim of the work was to examine whether the effectiveness of doxorubicin on these three breast cancer cell lines would be affected through the inhibition of Akt.

Six Akt inhibitors, alone or in combination with doxorubicin on human breast cancer cell lines were studied. All six inhibitors, which have different mechanisms to inhibit Akt, synergised the growth inhibitory effect of doxorubicin on MDA-MB-231 human breast cancer cell line, a human cancer cell line with mutated p53 and absent estrogen receptor. Two Akt inhibitors, II and IV, also synergised the growth inhibitory effect of doxorubicin on T47D human breast cancer cell line (mutant p53, ER^{+}), but others only induced additive effect on this cell line. In MCF-7 cells, which have wild-type p53 and are ER positive, inhibitors I, II and III, showed growth inhibitory effects when used alone, but did not enhance the effect of doxorubicin. It is important to point out that, as Akt inhibitors I, II and III are phosphatidylinositol ether analogues, it is inevitable that they all exhibit certain degree of inhibitory effect on PI3K activity. However, Akt I, II, III and indeed VIII have weak inhibition on PI3K. In addition, Akt inhibitor IV inhibits PKA, PKC and SGK and Akt inhibitor V has weak inhibitory effect on PKA, PKC, SGK, STAT3, p38, Erk1/2 and JNK. This finding may suggest that inhibition of one or more proteins mentioned above could sensitize MDA-MB-231 and T47D cell line to the effect of doxorubicin,

at the doses used in this study, this is unlikely. inhibitor II sensitised both cell lines to the effect of doxorubicin, whereas inhibitor III only sensitised the MDA-MB-231 cell line. The reasons for finding interactions between doxorubicin and some Akt inhibitors, but not with others, on the T47D cell line could be various. These inhibitors differ in solubility and pharmacokinetics. Lipid solubility plays an important role in determining drug bioavailability, and therefore the access to respective sites of action. On the other hand, a recent study showed that the inhibition of Akt using Akt inhibitor III sensitized antioestrogen-resistant MCF-7 sublines to the growth inhibitory effect of IGF-1 receptor-neutralizing antibodies, compared with the parental (10), suggesting hormone treatment resistant breast cancer patients might benefit from Akt inhibition treatment.

Furthermore, it is currently not known to what extent there is functional overlap between Akt1, Akt2 and Akt3. It has been reported that the HT 29 colon cancer cell line, the LnCaP human prostate cancer cell line, the A2780 human ovary cancer cell line and the MCF-7 human breast cancer cell line, but not non-malignant cell lines, could be sensitized to the effect of chemotherapeutics by Akt inhibitor VIII. This induction is not reversed by overexpression of functionally active Akt (11). It was also demonstrated in the same study that the inhibition of mTOR, a downstream substrate of Akt, is less effective in the induction of apoptosis compared with the inhibition of Akt, suggesting that the survival phenotype conferred by Akt may be mediated by signalling pathways independent of mTOR in some tumour cells. It remains to be determined which Akt isoenzyme inhibition profile will impact on cancer cell survival and minimize toxic effects (6,12). However, it is clear that the inhibition of Akt or a subset of Akt isoenzymes will benefit the treatment for cancer.

It has been recently reported that the combined treatment with Akt inhibitor VIII and TRAIL dramatically increased caspase-3 activity in LnCaP human prostate cancer cells (6- to 10-fold relative to control or TRAIL alone), and the increase in levels was similar when using either Akt inhibitor VIII (25 μM) or PI3K inhibitor LY294002 (15 μM) in combination with TRAIL (11,13). Furthermore, Akt inhibitor VIII, being non-competitive with ATP and peptide substrate, compared with other ATP-competitive Akt inhibitors, e.g., Akt inhibitor IV, is less likely to inhibit other members of the ACG family, such as PKA and PKC, and may show less non-specific cytotoxicity.

In terms of possible clinical use, Akt inhibitor V is the only one of the six Akt inhibitors used in this study that has been tested in a clinical trial. A phase I clinical trial of this inhibitor, which used a five-day continuous infusion schedule at 10-40 mg/m^2/day repeating every 3-6 weeks, showed limited anti-tumour activity on various cancers with multiple side effects, including hyperglycemia, hepatotoxicity, thrombocytopenia, anaemia, nausea and vomiting, etc. (14). A phase II clinical trial of Akt inhibitor V, which also used a five-day continuous infusion schedule at 35 mg/m^2/day repeating every 6 weeks on advanced squamous cell carcinoma of the cervix, also showed this inhibitor appears to have limited activity in metastatic or recurrent squamous cell carcinoma of the cervix with multiple side effects (15). Although it is not clear whether the cytotoxicity of Akt inhibitor V is related to the inhibition of Akt activation, it has been reported that the cytotoxicity is closely related to the dosage used in the trials (14,15). Due to the multiple significant side effects at the doses used in the clinical trials, this inhibitor has been limited in clinical use. Data presented in this study has demonstrated that low doses of Akt inhibitor V (at 62.5-1000 nM), which equals to 20-320 ng/ml) sensitised MDA-MB-231 human breast cancer cells to the effect of doxorubicin. It is difficult at this stage to directly compare the doses used in the clinical trials and the ones used *in vitro* in this study. However, it gives a clue that this inhibitor could be used at low doses in combination with other chemotherapeutics, instead of being used alone, to boost their anti-tumour activities, by which it is possible to minimize the side effects from both chemotherapeutics and Akt inhibitor V. Furthermore, it has been shown that the anti-tumour activity of Akt inhibitor V is selective for those tumours with aberrant Akt activity (16), suggesting further study on this inhibitor should incorporate subject selection considering the Akt status in the tumours.

So far, only very limited information on the efficiency and specificity of these inhibitors is available and little work has been done using them. These inhibitors are relatively new drugs, further investigations need to be carried out to optimise their pharmacokinetic properties and to understand their toxicity. Until the fundamental issue is solved, which means the development of more specific Akt inhibitors, the role that Akt plays in the sensitization of breast cancer cells to the effect of doxorubicin by using PI3K inhibitor LY294002 and whether Akt itself could be an effective target for cancer treatment will remain unclear.

Acknowledgements

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