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The efficacy and selectivity of tumor cell killing by Akt inhibitors are substantially increased by chloroquine

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ABSTRACT

This study was to evaluate the enhancement value of chloroquine (CQ) in cancer cell killing when used in combination with Akt inhibitors. The results showed that the combination of CQ and Akt inhibitors is much more effective than either one alone. Importantly, the CQ-mediated chemosensitization of cell killing effects by Akt inhibitors is cancer specific. In particular, when combined with 10 μM CQ, 1,3-dihydro-1-(1-((4-(6-phenyl-1*H*-imidazo[4,5-*g*]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2*H*-benzimidazol-2-one (an Akt1 and 2 inhibitor; compound **8**) killed cancer cells 10–120 times more effectively than normal cells. Thus, CQ is a very effective and cancer-specific chemosensitizer when used in combination with Akt inhibitors.

1. Introduction

Although overall survival rate of breast cancer patients has substantially increased during the last decades, this is mainly due to early tumor detection. Unfortunately, the recovery rate of advanced breast cancer by currently available treatment modalities is still unacceptably low. One of the promising modalities is to apply combinations of two or more different chemotherapeutic agents that can induce synergistic tumor cell killing.[1] Although such combinational therapies have shown to improve overall patient survival, they are often associated with treatment-related toxicity and the potential for developing cross-resistance to the drugs used.[2,3] Therefore, it is important to develop combined modalities that effectively kill tumor cells with minimal side-effects on normal cells.

The PI3K (phosphatidylinositol-3-kinase)–Akt pathway is involved in the regulation of multiple cellular processes including cell survival, proliferation, growth, angiogenesis, migration, and transformation.[4] Numerous reports have shown that constitutive activation of the PI3K–Akt signal pathway is closely correlated with tumor development, aggressive progression, increased metastasis, and resistance to cancer therapies.[5–13] In contrast, the PI3K–Akt pathway is tightly regulated to limit its activity in normal cells.[14] This difference in PI3K–Akt activities in normal and cancer cells can provide a way to effectively and specifically control cancer cells with a minimum cytotoxic effect on normal cells.

Inhibition of Akt alone or in combination with other tumor therapeutic agents resulted in the increase of programmed cell death, leading to radiosensitization, decreased tumor growth, anti-angiogenic and -tumorigenic, and decrease in tumor resistance to chemotherapy.[15–20] Unfortunately, inhibition of the PI3K pathway can cause undesirable side-effects.[21,22] Blocking downstream players in the PI3K–Akt pathway at low doses is likely less toxic, although the efficacy of such specific and narrow ranged inhibitors at low doses may be lower than upstream inhibitors at high doses. We hypothesized that the use of low doses of Akt inhibitors could be effective and safe if used in combination with appropriate chemosensitizers.

Chloroquine diphosphate (CQ) is a well-known anti-malarial drug possessing a 4-aminoquinoline scaffold. CQ is currently at clinical trials as an investigational anti-retroviral agent in humans with HIV-1/AIDS, who often develop tumors.[23–26] Recently, CQ has also been added to a conventional therapy protocol (i.e., surgery + radiotherapy + chemotherapy) for HIV-1 seronegative glioblastoma patients, which showed significant enhancement.[27]

CQ is uncharged at neutral pH and can freely diffuse across cell and organelle membranes. Once inside an acidic vesicle, however, CQ becomes protonated and can no longer easily diffuse out of the vesicle.[28] As a result, CQ molecules are trapped and accumulated in the cytoplasmic acidic vesicles such as lysosomes,[29] which may be relevant to the increase in lysosomal volumes and subsequent

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enlargement of the plasma membrane surface.[30,31] The increase in the lysosomal volume is a common event in apoptotic or necrotic cell death in response to diverse stimuli, including staurosporine and etoposide.[32] Furthermore, an increase in the lysosomal volume by either intrinsic or extrinsic factors can often change the mode of cell death from apoptosis to necrosis.[33] Elevation of the lysosomal pH may also influence endocytosis, exocytosis, and phagocytosis.[34–36]

CQ and its analogs may promote apoptosis in T-cells and C6 glioma cells. A recent report revealed that phosphatidylcholine-specific phospholipase C (PC-PLC) was involved in the death of L929 cells induced by TNF-α.[37] Furthermore, we have recently demonstrated that CQ substantially increases the cytotoxic effects on the metastatic MDA-MB231 breast cancer cell line when used in combination with radiation.[38]

The main objectives of this research are to examine (a) whether inhibiting the PI3K–Akt pathway with a pathway-specific blocker can specifically kill tumor cells and (b) whether CQ can effectively sensitize the cell killing effects of the PI3K–Akt blockers. We here report that PI3K–Akt inhibitors generally kill cancer cells more effectively than normal cells. Furthermore, CQ greatly sensitizes the cell killing effects of several Akt inhibitors in a cancer-specific manner.

2. Results

2.1. Cells and PI3K–Akt inhibitors

The cytotoxic effects of 11 PI3K–Akt inhibitors (Fig. 1) were examined for their cell killing effects in the absence or presence of CQ using MDA-MB468, MDA-MB231, and MCF-7 cancer cell lines. The PI3K–Akt inhibitors that were used in our experiments may be classified into five groups based on their different modes of action (Calbiochem/Merck KGaA, Darmstadt, Germany) (Fig. 1). The Group I is phosphatidylinositol analogs (compounds **1**, **2**, and **3**), which compete with phosphatidylinositol 4,5-bisphosphate (PIP_2) and, thereby, preventing the generation of phosphatidylinositol 3,4,5-triphosphate (PIP_3). The compounds in this group also compete with PIP_3 binding to Akt. The compounds **2** and **3** are metabolically more stable.[39,40] The exact molecular targets for the Group II inhibitors (compounds **4**, **5**, **9**, and **10**) are not well understood, although some of them apparently function downstream of PI3K or phosphoinositide-dependent kinases (PDKs).[41–44] The Group III includes two oligo peptides containing a TCL1-derived sequence (inhibitors **6** and **7**), which binds to the Akt PH domain and, thus, interfere the Akt–phosphoinositide interaction. These three groups interfere with the cellular activation of Akt; however, most of them do not affect Akt activities if already activated.[45,46] The Group IV inhibitors (compounds **8**, **9**, and **10**) are dependent upon a PH domain and, thus, are not functional in Akt lacking a PH domain or the closely related AGC (cAMP-dependent, cGMP-dependent, and protein kinase C) family kinases.[43,44,47] Importantly, the Group IV inhibitors can inhibit both the activation of Akt and its kinase activity. The compound **11** (LY294002) is cell permeable, potent, and specific (at low concentration) PI3K inhibitor that acts on the ATP-binding site of the enzyme. It also inhibits non-homologous end-joining repair through the inhibition of DNA-PK catalytic subunit.[48]

2.2. PI3K and Akt blockers efficiently inhibit cancer cell proliferation

As shown in Table 1, five of the eleven Akt inhibitors exhibited GI_{50} in the range of 1.12–8.68 μM on MDA-MB468 breast cancer cells (inhibitors **1**, **4**, **8**, **9**, and **10**). Four inhibitors showed GI_{50} ranging from 10.40 to 28.43 μM (inhibitors **2**, **6**, **7**, and **11**), and the rest above 20.80 μM (inhibitors **3** and **5**) on the same cancer cell line. It is noted that some of inhibitors showed substantially different growth inhibition effects on different cancer cell lines. The inhibitors **4**, **7**, **8**, **9**, **10**, and **11** showed GI_{50} ranging from 0.81 to 8.34 μM on MDA-MB231. In contrast, inhibitors **1**, **3**, and **5** required 24.30 μM or high concentrations to achieve GI_{50} on MDA-MB231, while the rest (inhibitors **2** and **6**) showed GI_{50} at 16.77 and 17.80 μM, respectively. As for MCF7 breast cancer cell line, the GI_{50} values of the inhibitors **4**, **5**, **8**, **9**, **10**, and **11** were 0.23–6.10 μM, and the rest (**1**, **2**, **3**, **6**, and **7**) at >16.01 μM.

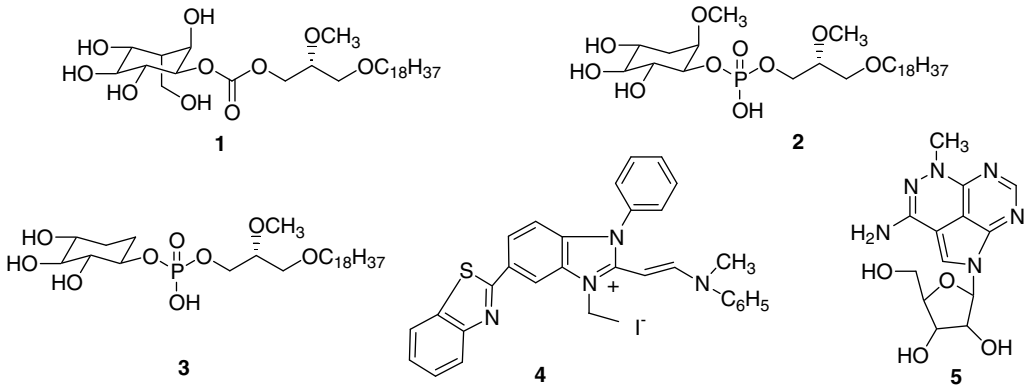
The compound **4** was the most effective on all three cancer cell lines, as its GI_{50} was 0.23, 0.81, and 1.74 μM on the MCF7, MDA-MB231, and MDA-MB468, respectively. The inhibitors **8**, **9**, **10**, and **11** were also quite efficient on all three cancer cell lines. Interestingly, the compound **1** was very effective on MDA-MB468, in which Akt is constitutively activated due to defect in PTEN, but not on MDA-MB231 and MCF7 cell lines (Table 1).

2.3. Chloroquine substantially enhances cell killing effects of PI3K and Akt inhibitors in a cancer-specific manner

The GI_{50} values of CQ were 28.58, 22.52, 38.44, and 76.13 μM on MDA-MB468, MDA-MB231, MCF7, and 184B5 cells, respectively (Fig. 2). This suggests that the cytotoxic effects of CQ on cells are generally low regardless of the origins of cell lines. The cell killing effect of CQ on 184B5, an immortalized 'normal' breast cell line,[49,50] was particularly low.

In spite of its inefficient cell killing, CQ could in most cases greatly increase the cell killing effects by Akt inhibitors. As shown in Table 1, 10 μM CQ increased the overall cell killing effects of Akt inhibitors by 32.2% (i.e., cell number decreased to 67.8% of the level shown by Akt inhibitor alone), 33%, and 25% on MDA-MB468, MDA-MB231, and MCF7, respectively. When the concentration of CQ increased to 20 μM, the sensitization effects increased by 65% (i.e., 100–35% = 65%), 66%, and 64% on MDA-MB468, MDA-MB231, and MCF7, respectively (Table 1). The enhancement rates of cell killing effects by CQ on some Akt inhibitors were very substantial. For example, the combination of 10 μM CQ with the Akt inhibitors **4** and **8** on MDA-MB468 resulted in the increase of cell killing by 64% and 58%, respectively, compared to that by Akt inhibitors alone. Similar enhancement effects by 10 μM CQ were also observed on MCF7 when combined with Akt inhibitors **5** (by 75%) and **8** (by 55%) (Table 1). In contrast, enhancement of cell killing effects by CQ on non-cancer cells was low. For example, the average enhancement of cell killing effects by 10 and 20 μM CQ on 185B5 was 11% and 24%, respectively (Table 2).

To gain further insights into the potential of CQ as an anticancer therapeutic agent, we assessed the CQ-mediated sensitization of cell killing effects by Akt inhibitors on cancer and non-cancer cells (Table 3). Most Akt inhibitors killed cancer cells more effectively than non-cancer cells, even in the absence of CQ. For example, Akt inhibitors **1**, **3**, **7**, **8**, **10**, and **11** killed cancer cells 1.32–11.41 times more efficiently than 184B5 (Table 3). Importantly, the differences in cell killing effects by Akt inhibitors on cancer and normal cells dramatically increased in the presence of CQ. For example, in the presence of 20 μM CQ, the cell killing of the inhibitors **1**, **3**, **5**, **7, 8**, and **11** on cancer cells was 6.88–78.42 times more effective than non-cancer cells. In average, the cell killing effects of the 11 PI3K/Akt inhibitors on cancer versus normal cells increased from 2.47-fold in the absence of CQ to 16.65-fold in the presence of 20 μM CQ (Table 3). Thus, the CQ-effect on differential enhancement of cell killing by PI3K–Akt inhibitors between cancer and normal cells is at least 6.7-fold.



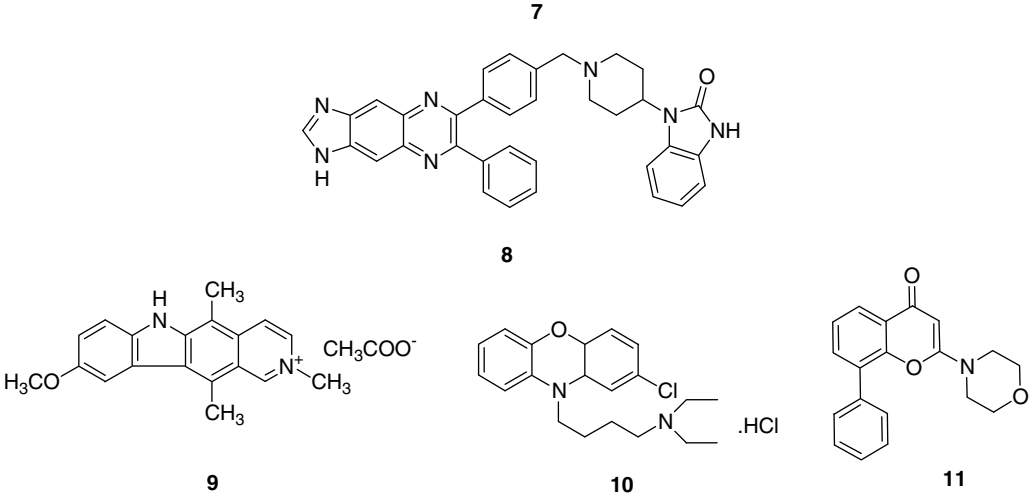
1 **2** **3** **4** **5**

H-Ala-Val-Thr-Asp-His-Pro-Asp-Arg-Leu-Trp-Ala-Trp-Glu-Lys-Phe-OH

6

H-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ala-Val-Thr-Asp-His-Pro-Asp-Arg-Leu-Trp-Ala-Trp-Glu-Lys-Phe-OH

7



8 **9** **10** **11**

Figure 1. The molecular structure of PI3K–Akt inhibitors is used in this study. The molecular structures of nine chemical compounds and amino acid sequences of two oligo peptides used in this work are shown. The inhibitors #**1**–**11** (left to right) are as follows (the numbers in brackets are Calbiochem catalog numbers): **1** L-6-hydroxymethyl-*chiro*-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (124005); **2** phosphoric acid 2-methoxy-3-octadecyloxy-propyl ester 2,3,4-trihydroxy-6-methoxy-cyclohexyl ester (124008); **3** phosphoric acid 2-methoxy-3-octadecyloxy-propyl ester 2,3,4-trihydroxy-cyclohexylester (124009); **4** 6-benzothiazol-2-yl-1-ethyl-2-[2-(methyl-phenyl-amino)-vinyl]-3-phenyl-3*H*-benzoimidazol-1-ium iodide (124011); **5** 2-(3-amino-5-methyl-5*H*-1,4,5,6,8-pentaaza-acenaphthylen-1-yl)-5-hydroxymethyl-tetrahydro-furan-3,4-diol; **6** H-AVTDHPDRLWAWEKF-OH $TCL1_{10-24}$ (124013); **7** H-YGRKKRRQRRR-AVTDHPDRLWAWEKF-OH TAT-$TCL1_{10-24}$ (124014); **8** 1-{1-[4-(7-phenyl-1*H*-imidazo[4,5-*g*]quinoxalin-6-yl)-benzyl]-piperidin-4-yl}-1,3-dihydro-benzoimidazol-2-one (124018); **9** 9-methoxy-2,5,11-trimethyl-6*H*-pyrido[4,3-*b*]carbazol-2-ium acetate (124019); **10** [4-(2-chloro-4a,10a-dihydro-phenoxazin-10-yl)-butyl]-diethyl-amine hydrochloride (124020); **11** 2-morpholin-4-yl-8-phenyl-chromen-4-one (LY294002).

3. Discussion and conclusion

We previously reported that CQ accumulated in the lysosomes, by which the lysosomal membrane was destabilized. We also found that ceramide was accumulated in the lysosomes of the cells treated with both CQ and ionizing radiation, but not in the cells treated with either one only.[38] The high levels of ceramide in the lysosomes by a combination of radiation and CQ further destabilized the lysosomal membrane. Importantly, 10 μM CQ alone killed only a fraction of cells, but effectively sensitized radiation-mediated cell killing.[38]

Here, we report that CQ also effectively sensitizes the cell killing effects of PI3K–Akt inhibitors, suggesting that CQ has the potential to significantly enhance both radiation and chemotherapy. We found that the average GI_{50} value of CQ on three cancer cell lines (MDA-MB231, MDA-MB468, and MCF7) was 29.84 μM, and that on the non-cancer 184B5 breast cell line was 76.13 μM (Fig. 2). These data demonstrate that CQ does not effectively kill either cancer or non-cancer cells. In particular, the cytotoxic effects of CQ on non-cancer cells are very low. However, CQ can greatly enhance the cell killing effects by PI3K–Akt inhibitors. The average enhancement of 20 μM CQ on cancer cell killing by PI3K–Akt inhibitors was 16.65-fold (Table 3). Together, our data suggest that CQ has the potential of significantly enhancing both radiation and chemotherapy, as it can increase cancer cell killing much more effectively than non-cancer cells by Akt inhibitors.

We have demonstrated in this report that the Group IV Akt inhibitors (compounds **8**–**10**) and LY294002 (PI3K inhibitor; compound **11**) are generally more effective than other groups. This may be at least in part due to the fact that this Akt subgroup can directly bind

Table 1
Cytotoxicity of the compounds **1**–**11** in the absence or presence of CQ[a]

Inhibitors[b]	GI_{50} (μM)[c]									
	MDA-MB468[d]			MDA-MB231[d]			MCF7[d]		
	I[e]	I + CQ[f]	I + CQ[g]	I[e]	I + CQ[f]	I + CQ[g]	I[e]	I + CQ[f]	I + CQ[g]
1	2.88 ± 0.02 (100%)[h]	2.01 ± 0.02 (70%)	1.81 ± 0.01 (63%)	24.30 ± 0.81 (100%)	18.04 ± 0.75 (74%)	0.31 ± 0.01 (1%)	28.88 ± 0.91 (100%)	27.23 ± 0.85 (94%)	6.38 ± 0.07 (22%)
2	28.43 ± 1.05 (100%)	19.26 ± 0.12 (68%)	0.06 ± 0.01 (2%)	16.77 ± 0.72 (100%)	14.67 ± 0.62 (87%)	9.39 ± 0.08 (56%)	16.01 ± 0.89 (100%)	15.64 ± 0.62 (98%)	7.94 ± 0.08 (50%)
3	32.38 ± 1.15 (100%)	26.60 ± 1.01 (82%)	0.06 ± 0.01 (0.2%)	25.69 ± 0.89 (100%)	15.00 ± 0.59 (58%)	0.63 ± 0.03 (3%)	16.45 ± 0.91 (100%)	15.91 ± 0.63 (97%)	5.50 ± 0.06 (33%)
4	1.74 ± 0.01 (100%)	0.63 ± 0.05 (36%)	0.36 ± 0.01 (21%)	0.81 ± 0.07 (100%)	0.69 ± 0.03 (85%)	0.57 ± 0.01 (70%)	0.23 ± 0.02 (100%)	0.20 ± 0.01 (87%)	0.12 ± 0.01 (52%)
5	43.53 ± 1.56 (100%)	20.45 ± 0.08 (47%)	10.29 ± 0.09 (24%)	50.15 ± 1.86 (100%)	37.00 ± 1.02 (74%)	0.69 ± 0.04 (1%)	3.64 ± 0.06 (100%)	0.56 ± 0.04 (25%)	0.05 ± 0.01 (1%)
6	19.95 ± 0.68 (100%)	15.02 ± 0.65 (75%)	9.98 ± 0.08 (50%)	17.80 ± 0.67 (100%)	14.37 ± 0.56 (81%)	8.21 ± 0.23 (46%)	23.45 ± 1.12 (100%)	16.52 ± 0.89 (70%)	8.52 ± 0.18 (36%)
7	20.80 ± 0.71 (100%)	16.89 ± 0.67 (81%)	11.25 ± 0.12 (54%)	5.99 ± 0.05 (100%)	3.99 ± 0.02 (67%)	1.21 ± 0.01 (20%)	45.58 ± 1.86 (100%)	32.56 ± 1.23 (71%)	25.32 ± 0.98 (56%)
8	6.30 ± 0.04 (100%)	2.66 ± 0.03 (42%)	0.85 ± 0.02 (13%)	5.31 ± 0.05 (100%)	4.55 ± 0.02 (86%)	2.95 ± 0.04 (55%)	0.92 ± 0.01 (100%)	0.41 ± 0.02 (45%)	0.05 ± 0.01 (5%)
9	1.12 ± 0.01 (100%)	0.78 ± 0.01 (70%)	0.75 ± 0.02 (67%)	5.90 ± 0.04 (100%)	3.74 ± 0.01 (63%)	1.99 ± 0.05 (34%)	1.00 ± 0.04 (100%)	0.59 ± 0.03 (59%)	0.56 ± 0.05 (56%)
10	8.68 ± 0.05 (100%)	8.37 ± 0.06 (96%)	3.95 ± 0.05 (45%)	8.34 ± 0.08 (100%)	5.69 ± 0.03 (68%)	3.63 ± 0.08 (44%)	6.10 ± 0.08 (100%)	5.08 ± 0.05 (83%)	1.78 ± 0.04 (5%)
11	10.40 ± 0.09 (100%)	8.20 ± 0.05 (79%)	4.76 ± 0.07 (46%)	6.71 ± 0.08 (100%)	4.35 ± 0.06 (65%)	3.32 ± 0.05 (49%)	3.16 ± 0.05 (100%)	3.08 ± 0.04 (97%)	2.63 ± 0.08 (83%)
Average survival	100%	67.8%	35%	100%	67%	34%	100%	75%	36%

[a] Calculation was from sigmoidal dose–response curves (variable slope) that were generated using GraphPad Prism V. 4.02 (GraphPad Software Inc.).
[b] Details of the 11 Akt inhibitors are shown in Figure 1.
[c] GI_{50}, concentration of drug needed to reduce cell growth (i.e., cell number) to 50% of the untreated control level; values are mean of triplicates of at least three independent experiments.
[d] Human breast cells or adenocarcinoma cell lines.
[e] Cell cytotoxicity of an Akt inhibitor alone.
[f] Cell cytotoxicity of an Akt inhibitor plus 10 μM of CQ.
[g] Cytotoxicity of an Akt inhibitor plus 20 μM of CQ.
[h] Cell survival fraction after the treatment of an Akt inhibitor in the absence of CQ is calculated as 100%.

to either non-activated or activated Akt, thereby, inhibiting both the activation of Akt and the kinase activity of Akt. Furthermore, this group has distinct advantage in selectively killing cancer cells with high level of Akt, since these compounds inhibit Akt in a PH-domain dependent manner. The high efficacy of the compound **11** is expected since it is a broader inhibitor of the PI3K–Akt pathway than the rest.

The cytotoxic effect of the compound **4** was the most pronounced among the 11 inhibitors used in this study (Table 1). However, this compound also very effectively killed non-cancer cells (Table 2). Furthermore, the enhancement of cell killing by the combination of compound **4** and CQ was not substantially different for cancer and non-cancer cells (Table 3). Therefore, compound **4** may not be an ideal anticancer therapeutic agent.

Considering the overall efficacy on both cell killing and differential sensitization by CQ on cancer and normal cells, inhibitors **8** and **11** appear to be the best among the 11 PI3K–Akt inhibitors. The compound **8** is particularly promising, as it can kill all three cancer cells very effectively (GI_{50}, 6.30 ± 0.04, 5.31 ± 0.05, and 0.92 ± 0.01 on MDA-MB468, MDA-MB231, and MCF7, respectively). Furthermore, when combined with CQ, the compound **8** can effectively kill cancer but not normal cells. For instance, the GI_{50} values of the compound **8** in combination with 10 μM CQ are 2.66, 4.55, 0.41, and 50.58 μM, respectively, on MDA-MB468, MDA-MB231, MCF7, and 184B5 (Tables 1 and 2). These data suggest that the compound **8** in combination of 10 μM CQ can kill cancer cells 11.11 (MDA-MB231), 19.02 (MDA-MB468), and 123.36 (MCF7) times more effectively than non-cancer cells.

The exact mechanism how CQ differentially and specifically sensitizes cancer cell killing by PI3K–Akt inhibitors is presently unknown. However, it should be noted that the PI3K–Akt signal pathway is elevated in cancer cells, but not in normal cells. Therefore, it is possible that the impediment of normal lysosomal function by CQ results in further downregulation of the PI3K–Akt survival signal pathway.

Overall, our data raise the possibility that CQ can significantly increase the therapeutic effects of PI3K–Akt inhibitors with a minimal side-effect on normal cells. The combination of the compound **8** with a low concentration of CQ appears to be a particularly promising anticancer modality.

4. Experimental

4.1. Cell lines

The human MDA-MB468, MDA-MB231, and MCF7 breast cancer cell lines were maintained in RPMI 1640 medium (Fisher Scientific, Ottawa, Ont., Canada) supplemented with 10% fetal bovine serum (Hyclone, Logan UT) and 2 mM L-glutamine. Cells were grown at 37 °C with 5% CO_2, 95% air under the humidified conditions.

4.2. Reagents

CQ and LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) were purchased from Sigma–Aldrich Canada Ltd (Oakaville, Ont., Canada). Akt inhibitors (Fig. 1) were purchased from Calbiochem (purchased through VWR CANLAB, Mississauga, Ont., Canada). CQ and Akt inhibitors were dissolved in <0.1% dimethyl sulfoxide (DMSO) and diluted in culture medium (0.1–100 μM) immediately before use. The final concentration of DMSO in sulforhodamine B (SRB)-based cytotoxicity assays did not exceed 0.1%. To rule out that the DMSO concentration used does not affect cell cytotoxicity measurement, culture medium containing equivalent concentration of DMSO was used as a negative control in all experiments. In all studies, the concentration of DMSO used did not affect cell cytotoxicity.

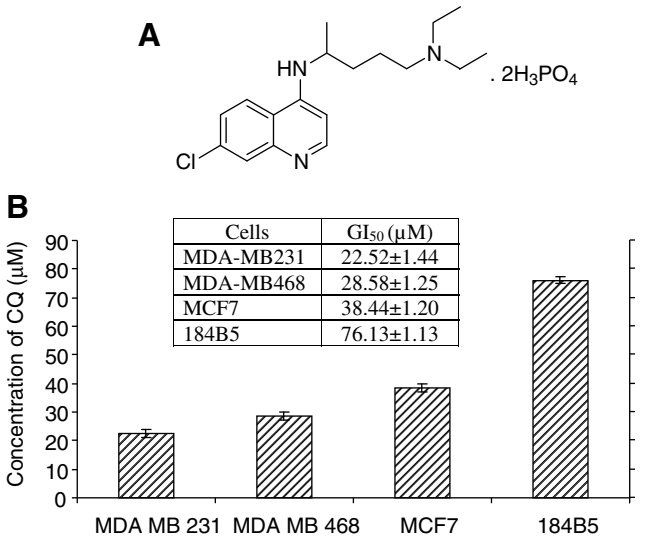


Figure 2. The cytotoxic effect of CQ is very low in non-cancer cells. (A) The molecular structure of CQ. (B) GI_{50} of CQ on three breast cancer cell lines and one non-cancer cell line (185B5) are shown in a diagram as well in a table format.

Table 3
CQ differentially sensitizes cell killing effects of Akt inhibitors on normal and cancer cells

Compound[b]	GI_{50} of 184B5/GI_{50} of MDA-MB231 (fold)[a]	
	No. of CQ[c]	Plus 20 μM CQ[d]
1	1.32 (31.85/24.03)	78.42 (24.31/0.31)
2	0.82 (13.76/16.77)	0.96 (8.98/9.39)
3	1.76 (45.25/25.69)	49.10 (30.93/0.63)
4	1.07 (0.87/0.81)	1.35 (0.77/0.57)
5	0.78 (40.00/50.15)	24.58 (16.96/0.69)
6	0.69 (12.22/17.80)	0.97 (7.93/8.21)
7	2.17 (13.00/5.99)	6.88 (8.32/1.21)
8	11.41 (60.60/5.31)	8.73 (25.76/2.95)
9	0.06 (0.36/5.90)	0.26 (0.52/1.99)
10	1.24 (10.38/8.34)	3.01 (10.94/3.63)
11	5.87 (39.37/6.71)	8.85 (29.37/3.32)
Average	2.47	16.65

[a] The 50% growth inhibition values of each Akt inhibitor on normal and cancer cells (Tables 1 and 2, respectively) were compared by dividing the GI_{50} value of normal cells with that of MDA-MB231 cancer cells.
[b] For detail, see Figure 1.
[c] Differential cytotoxic effects of each Akt inhibitor on cancer and non-cancer cells in the absence of CQ.
[d] The same as '[c]' but in the presence of 20 μM CQ.

Table 2
The cytotoxic effects of the compounds **1**–**11** on the 185B5 non-cancer cell line in the absence or presence of CQ[a]

Inhibitors[b]	GI_{50}(μM)[c]		
	184B5[d]		
	I[e]	I + CQ[f]	I + CQ[g]
1	31.85 ± 1.06 (100%)[h]	28.03 ± 0.71 (88%)	24.31 ± 0.65 (76%)
2	13.76 ± 0.46 (100%)	10.35 ± 0.31 (75%)	8.98 ± 0.25 (65%)
3	45.25 ± 1.35 (100%)	39.37 ± 1.25 (87%)	30.93 ± 1.01 (68%)
4	0.87 ± 0.05 (100%)	0.72 ± 0.04 (83%)	0.77 ± 0.04 (89%)
5	40.00 ± 1.56 (100%)	34.00 ± 1.11 (85%)	16.96 ± 0.67 (42%)
6	12.22 ± 0.08 (100%)	9.54 ± 0.10 (78%)	7.93 ± 0.12 (65%)
7	13.00 ± 0.09 (100%)	11.42 ± 0.11 (88%)	8.32 ± 0.24 (64%)
8	60.60 ± 1.68 (100%)	50.58 ± 1.54 (83%)	25.76 ± 0.87 (43%)
9	0.36 ± 0.01 (100%)	0.47 ± 0.04 (130%)	0.52 ± 0.05 (144%)
10	10.38 ± 0.05 (100%)	10.63 ± 0.24 (102%)	10.94 ± 0.56 (105%)
11	39.37 ± 1.32 (100%)	32.37 ± 1.21 (82%)	29.37 ± 1.05 (75%)
Average	100%	89%	76%

[a–h]For detail, see legend to Table 1.

4.3. SRB assay

Cytotoxic effects of CQ and Akt inhibitors were determined by an SRB-based test.[51] For a typical screening experiment, 5000–10,000 cells were inoculated into 100 μl medium per well of a 96-well microtiter plate as described previously.[52] Briefly, after the inoculation, the microtiter plate was incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. Some of the sample wells were fixed with 25 μl of 50% trichloroacetic acid (TCA) as a control of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs (10 mM) solubilized in DMSO were stored frozen. At the time of drug addition, an aliquot of the frozen stock was thawed and diluted twice to the desired final maximum test-concentration with complete medium. Two- to 10-fold serial dilutions were made to provide a total of seven drug concentrations. Following addition of drugs, the culture plate was incubated for additional 48 h. Cells were fixed in situ by slowly adding 25 μl of cold 50% (w/v) TCA (final concentration, 10% TCA), and were then incubated for 60 min at 4 °C. The supernatant was discarded, and the plate was washed five times with tap water, followed by air-drying. Fifty microliters of SRB solution at 0.4% (w/v) in 1% acetic acid were added to each well, and the plate was incubated for 10 min at room temperature. Unbound SRB was removed by washing the plate five times with tap water, followed by air-dry. The cells 'stained' with SRB were solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515–564 nm. Using nine-absorbance measurements [time zero (T_z), control growth (C), and test growth in the presence of drug at the seven concentration levels (T_i)], the growth rate (%) was calculated at each of the drug concentrations.

The relative absorbance values $(T_i - T_z)/(C - T_z)$ of each concentrations of experimental drug were generated for dose–response analysis. The dose–response parameters were calculated for each experimental agent. The 50% inhibitory concentration values for the compounds were obtained from the dose–response curves using non-linear dose–response curve fitting analyses with GraphPad Prism V. 4.03 software. Values were calculated for each of these parameters if the level of activity was reached. However, if the effect was not reached or was exceeded, the value for that parameter was expressed as greater or lesser than the maximum or minimum concentration tested.[51]

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