Primary and Secondary Resistance to Tyrosine Kinase Inhibitors in Lung Cancer

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Abstract. *Background: Tyrosine kinase inhibitors (TKI) have emerged as important therapeutic agents for the treatment of several types of cancer including lung cancer. Recent research attempts show that only a small population of cancer patients responds to TKI and furthermore, these patients eventually develop resistance. Studies support the classification of resistance in primary and secondary resistance. Materials and Methods: In the present study the differentiation between primary and secondary resistance to TKI in lung cancer cell lines was investigated. Lung cancer cell lines were tested for viability, apoptosis and cell cycle after exposure to the TKI erlotinib and gefitinib. Results: Cells with primary resistance showed similar cell-cycle patterns to those with secondary resistance but differences were observed between the two groups in the viability and apoptosis assays. Conclusion: Understanding the effects of TKI on cell signaling pathways would shed light on the mechanisms of acquired resistance and the differences between primary and secondary resistance.*

Tyrosine kinase inhibitors are novel therapeutics which are applied in the treatment of lung cancer. These types of drugs target specific molecules of the cell through which signaling pathways are blocked, hence preventing the growth of tumor cells. By specifically targeting certain molecules of the cell, non-cancerous cells are normally not affected which eventually leads to reduced side-effects in patients undergoing

Abbreviations: Er: Erlotinib; Ge: Gefitinib; TKI: Tyrosine kinase inhibitors.

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treatment (1, 2). Because of this, the use of targeted therapy approaches, such as TKI, present a preferred way of cancer treatment compared to traditional chemotherapy (3).

Despite having fewer side-effects TKI are still not optimal in the treatment of cancer; these drugs are mostly applied as 2nd or 3rd line therapies after failure of chemotherapy (4-6). Some of the reasons why TKI are not effective are: high selective responsiveness of patients and resistance development after exposure to the drugs (7).

It has been reported that some patients respond very well to TKI whereas some do not respond at all when they first receive treatment (8-10). It has also been observed that those who respond to TKI eventually, after continuous exposure, develop resistance to the drugs (11, 12). Considering these two categories of patients, resistance to TKI can be said to be either primary, meaning that the patients do not respond at all to treatment or secondary, meaning that patients respond to treatment at the beginning but develop resistance later on.

Patients who respond well have been identified as those who harbor mutations in the epidermal growth factor receptor (*EGFR*) gene (8, 13-16) whereas those who do not respond are those who carry a wild-type *EGFR* and/or *KRAS* mutations (12, 17, 18). TKI normally target the tyrosine domain of the EGFR by competing for ATP binding sites which subsequently prevents phosphorylation of the EGFR. This blocks the EGFR downstream signaling pathway, hence preventing the growth of cancer cells. Some of the activating mutations related to this mechanism include: L858R in exon 21, V765A and T783A in exon 20 deletions in exon 19 such as ΔLRE, (8, 19). These mutations are referred to as activating mutations because they enhance the activity of the kinase, hence increasing the affinity of the drugs to the EGFR.

Secondary resistance has, therefore, been associated with mutations in the *EGFR* gene. The T970M mutation has been reported to occur in patients undergoing TKI therapy and has been related to acquired resistance (10, 20, 21). Besides mutations in the *EGFR*, *MET* amplification has also been identified as one of the causes of acquired resistance to TKI

(21, 22). Primary resistance on the other hand has been associated with lack of activating mutations in the *EGFR* and active multi-drug resistant genes or active ABC transporters.

In the present study we sought to compare primary and secondary resistance to the TKI, erlotinib and gefitinib in lung cancer cells. Erlotinib and gefitinib are the most commonly used TKI in the treatment of lung cancer and were some of the very first TKI to be approved for patient use. Although several studies have been carried-out to establish the causes of resistance to these two drugs, there is not much information linking primary resistance to secondary resistance. Furthermore, most studies have concentrated on the mechanisms of secondary resistance to TKI rather than primary resistance.

Therefore, the aim of the present study was to compare lung cancer cells with primary resistance to those with secondary resistance after exposure to erlotinib and gefitinib. This was performed to establish similarities in the mode of action of the drugs on cells with primary and secondary resistance.

Materials and Methods

Study scheme. Lung cancer cell lines A549 (DSMZ), H1299 (ATCC), and HCC827 (DSMZ), were used in this study. In a cell culture model, HCC827 cells were used to simulate secondary resistance. This was performed by culturing the cells exposed to the test substances for a long period of time (approximately 3 months). In short, the cells were seeded in cell culture flasks and then erlotinib and gefitinib (LC Labs, Woburn, MA, USA) were added at concentrations of 1 μM and 10 μM. Cell culture medium was changed every 3rd day and drugs were added to the fresh medium. The cells were only split after reaching confluence. Treated cells were compared to controls and resistance was thought to have occurred when treated cells reached confluence at the same time as the control. Apart from the original cell line (which is referred to as the parent cell line re-named HCC827P) two other sub-cell lines were derived and these were renamed HCC827ErR (resistant to erlotinib), HCC827GeR (resistant to gefitinib).

Cell culture. A549 was cultured in DMEM with 1% pen/strep and 10% FBS, H1299 in RPMI with 1% pen/strep and 10% FBS whereas HCC827 was cultured in RPMI with 1% pen/strep and 15% FBS. The cells were washed 1x with PBS w/o Ca+ Mg+ and then trypsized (A549 and H1299) or treated with TrypLE (Invitrogen, Carlsbad, CA, USA) (HCC827) at 37°C for 3 min. The reaction was then stopped by adding 10 ml cell culture medium and then the cells were centrifuged at $300 \times g$. The cells were then re-suspended in 10 ml culture medium and counted in a neubauer chamber after staining with 4% trypan blue (Invitrogen, Carlsbad, CA, USA). The cells were seeded in 96 well plates for the viability and apoptosis assays at a density of 1×10^4 cells per well. For cell-cycle analyses, cells were seeded in 6-well plates at a density of 2.5×10^5 cells per well. Twenty-four hours later the medium was changed and the drugs were added to the cells. The cells were then further incubated with the drugs at 37°C, 5% CO_2 before carrying out the various tests. The reagents used above were obtained from Biochrom (Biochrom, Berlin, Germany) unless stated otherwise.

Viability tests were carried out to establish the sensitivity of lung cancer cells to erlotinib and gefitinib. The cells were exposed to 0.01 μM – 100 μM of erlotinib and gefitinib and then the MTT assay test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was carried out at 48 h, 72 h and 96 h. The MTT reagent (Sigma Aldrich, Taufkirchen, Germany) was added to each well at a ratio of 1:10 and the cells incubated at 37°C for 1 h prior to measuring the absorbance on the tecan plate reader at 650 nm. After establishing the sensitivity of the cells to the drugs apoptosis assays were carried out to find out if the drugs indeed induced apoptosis. The ApoOne caspase 3/7(Promega, Mannheim, Germany) assay was carried out 48 h after exposure of the cells to the drugs. The ApoOne reagent was added to each well at a ratio of 1:1 followed by incubation for 1 h at RT. The luminescence was then measured on the tecan plate reader. Finally cell-cycle analysis was carried out to establish if the drugs induced a cell-cycle arrest in the lung cancer cells. The cells were fixed in ice-cold ethanol, after trypsinization the cells were washed 1× with PBS and then re-suspended in 500 μl PBS, followed by addition of 1.5 ml ice-cold ethanol. To stain the cells, they were centrifuged and then the ethanol aspirated before adding 500 μl of propidium iodide buffer (BD, Heidelberg, Germany). FACS analysis was carried out with the Beckman Coulter® FC500 equipment. The cells were exposed to the test substances 48 h prior to the FACS analysis.

Statistical analysis. Statistical analyses were carried out for each experiment, three independent trials were included. The collected data were summarized in Excel worksheets and basic statistical analyses carried out. Further statistical analyses and graph drawing were done using SigmaPlot 11.0. Analysis of variance (Holm sidak test) was carried out to compare three groups of cells in order to determine their sensitivity to erlotinib and gefitinib. Significance levels were established between the individual cell lines to their untreated control as well between the different cell lines.

Results

Sensitivity of A549, H1299 and HCC827 cells to erlotinib and gefitinib. Lung cancer cells A549, H1299 and HCC827 were tested for sensitivity against erlotinib and gefitinib. Figure 1 shows the effect of the drugs on cell growth at different incubation periods. Erlotinib and gefitinib showed a concentration-dependent growth inhibition of A549 cells (the cell growth reduced with increasing drug concentration). Furthermore, cell growth was reduced by approximately 20% between the lowest (0.01 μM) and the highest (100 μM) concentrations for both drugs apart from gefitinib-treated cells at 96 h, where a 40% decrease was observed. At 96 h, gefitinib showed the highest growth inhibition whereas erlotinib had the highest effect on growth after 72 h. Erlotinib showed gradual cell growth reduction with increasing drug concentration at 48 h and 72 h but not at 96 h, whereby growth reduced only at lower concentrations (<1 μM) and then the cell number again rose at the 1-μM concentration. Gefitinib, on the other hand, showed gradual cell reduction in cell number at 72 h. At 48 h cell growth remained constant between 1 μM and 100 μM. At 96 h there was a drastic reduction in cell number between 10 μM and 100 μM.
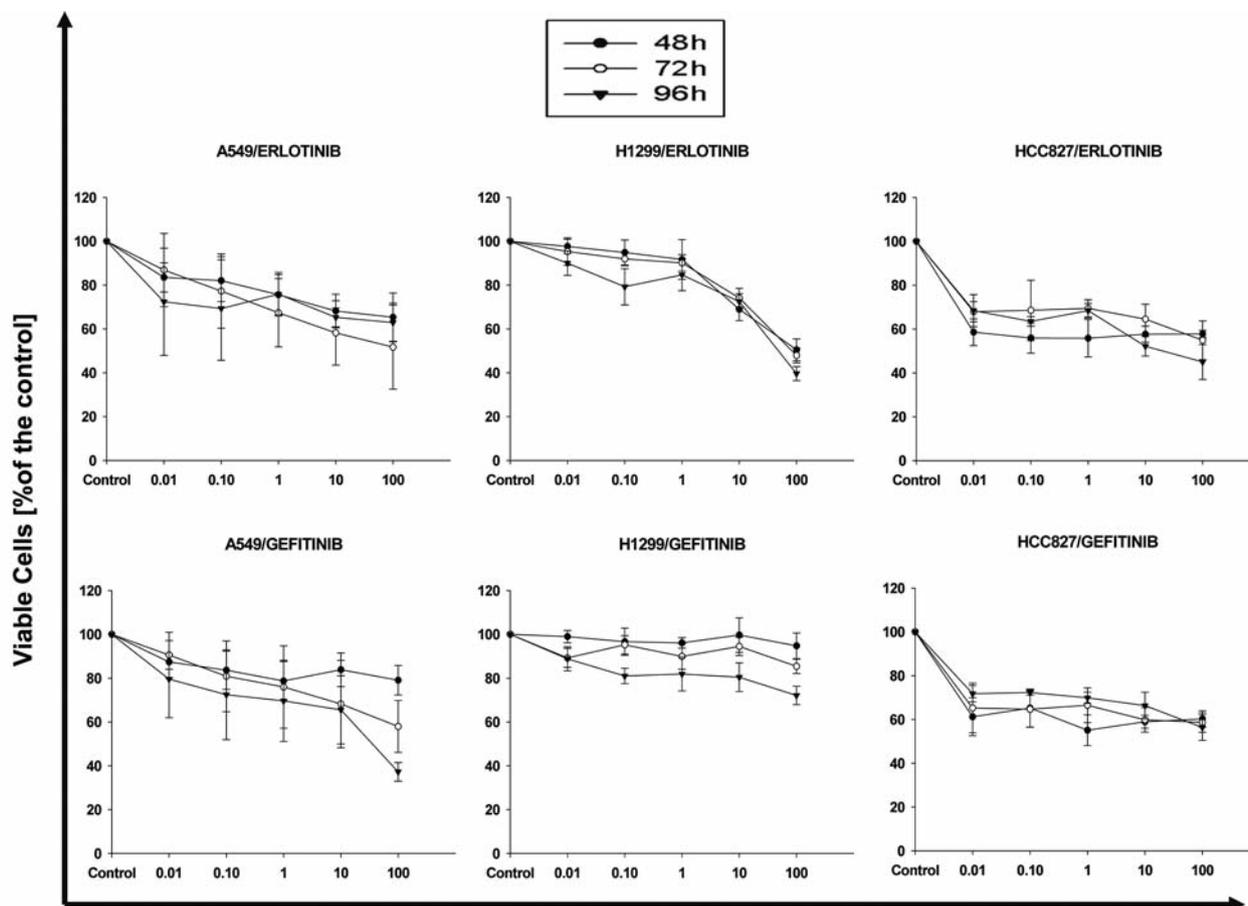
Figure 1. *Concentration response curves of lung cancer cells after exposure to erlotinib and gefitinib. Cell viability was assessed using the MTT assay and measurements were taken at 48 h, 72 h and 96 h after treatment with different concentrations of erlotinib and gefitinib.*

A concentration-dependent growth inhibition of erlotinib was also observed in H1299 cells and, furthermore, higher concentrations led to an increased growth inhibition. Erlotinib-treated cells also showed gradual reduction in cell growth at lower concentrations and a rapid decrease between 1 μM and 100 μM. The difference in the cell growth between 0.01 μM and 100 μM was 40%. Gefitinib-treated cells on the other hand showed a decrease in cell growth at 0.01 μM after 72 h and 96 h incubation period but not at 48 h and this remained almost constant for all concentrations applied. About 10% growth differences were observed between the lowest and highest concentrations at 96 h but not at 48 h and 72 h. The different incubation time showed similar trends in cell growth. However, the lowest cell growth was observed at 96 h in erlotinib-treated cells as well as gefitinib-treated cells. The cell growth dropped drastically in erlotinib-treated cells, at 10 μM and 100 μM after 48 h, 72 h and 96 h incubation periods. The cell growth in gefitinib-treated cells on the other hand was gradual. It was observed that erlotinib had a higher cytotoxicity effect on the cells than gefitinib.

Erlotinib and gefitinib inhibited growth of HCC827 cells, however an increase of the dose did not show increased growth inhibition. Cell growth dropped significantly at 0.01 μM (by about 40%) and then remained constant at increasing concentration; this was observed in erlotinib-treated cells as well as gefitinib-treated cells. Differences in cell growth between the lowest drug concentration and the highest drug concentration were not observed. The cells were more sensitive to erlotinib than gefitinib however differences were not significant ($p>0.05$). Similar trends in cell growth were observed at all incubation periods. Further incubation did not increase the rate of growth inhibition; the highest inhibition was recorded at 48 h and not 96 h as reported in A549 and H1299.

Statistical analyses of the end-point measurements were carried-out and the results are presented in Figure 2. For erlotinib-treated cells, significant differences were observed in A549 and HCC827 cells at all concentrations ($p<0.05$). In HCC827 cells, however, the differences were greater in HCC827 than in A549. This was observed at all
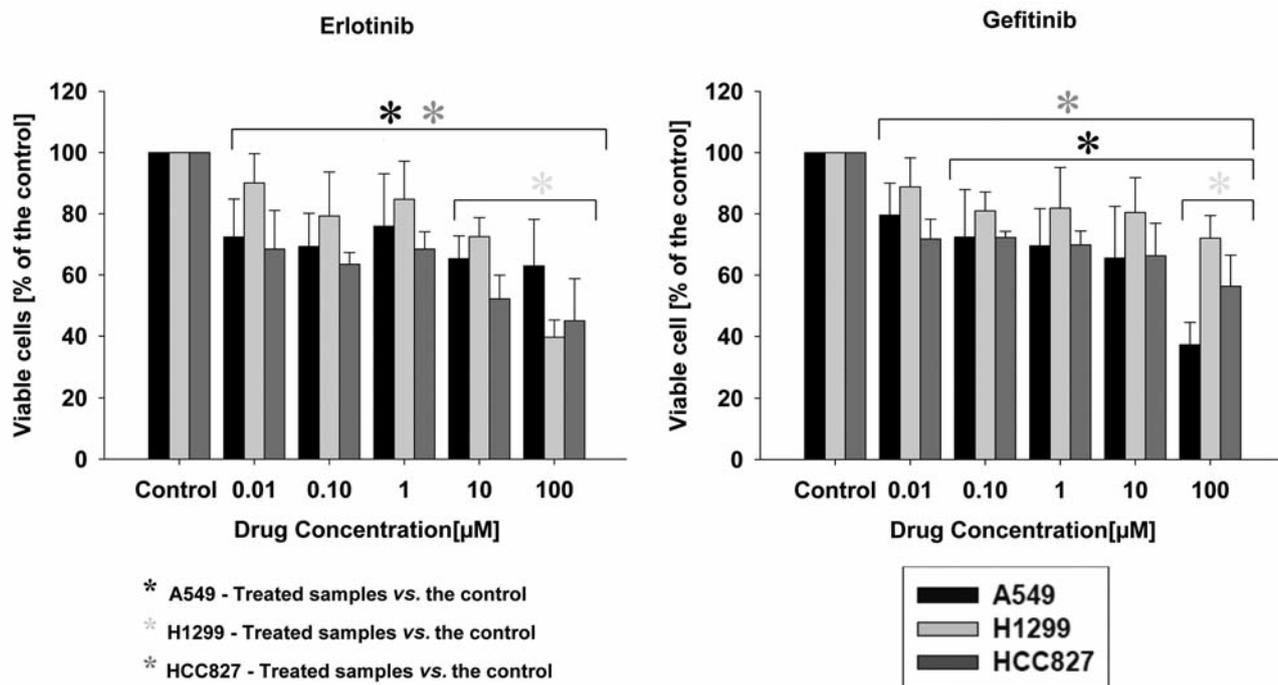
Figure 2. *Growth inhibition of erlotinib and gefitinib of A549, H1299 and HCC827 cells at 96 h. Results are expressed as the percentage of the controls.*

concentrations ($p<0.001$). H1299 cells on the other hand only showed significant differences at 10 μM ($p=0.004$) and 100 μM ($p=0.001$). However these cells (H1299), showed the highest growth inhibition at 100 μM. The effects of erlotinib on A549 and HCC827 cells were comparable at 96h, however the concentration response curves in Figure 1 and showed great differences between the two cells lines at different incubation points.

Gefitinib-treated cells showed significant differences between the treated samples and the control in A549 and HCC827 cells at all concentrations ($p<0.05$) apart from 0.01 μM in A459 cells ($p=0.056$). Furthermore, significant differences were only observed at 100 μM ($p=0.003$) in H1299 cells whereas the rest of the concentrations did not show significant differences to the control ($p>0.05$). A549 cells showed the highest growth inhibition when treated with 100 μM of gefitinib. Generally, similar observations were made for gefitinib-treated cells as well as in erlotinib-treated cells, whereby the growth inhibition of gefitinib in A549 and HCC827 cells was comparable at all concentrations apart from at 100 μM where A549 cells showed the least cell growth.

Sensitivity of HCC827 cells to erlotinib and gefitinib after developing resistance. From the results presented in Figure 1, it was seen that HCC827 cells were more sensitive to the test substances than A549 and H1299 cells. Because of their

sensitivity, HCC827 cells were used to simulate secondary resistance by exposing them to the drugs. By doing this it was possible to select the resistant cells from the sensitive cells. The resistant cells were later tested for viability after exposure to the test drugs as explained in the previous section. Figure 3 shows the effect of erlotinib and gefitinib on the growth of the resistance sub-cell lines.

There was a dose-dependent growth inhibition of erlotinib in all the cells. However, differences were observed between the parent and resistant cells. In HCC827P the cell growth decreased drastically at 0.01 μM but increasing concentrations showed a gradual decrease in cell growth. This was observed at 48 h and 72 h but not at 96 h whereby the decrease in cell growth was gradual at all concentrations, reducing with increasing drug concentration. It was observed that growth of the resistant HCC827ErR and HCC827GeR cells increased at 0.01 μM and then decreased gradually with increasing drug concentrations.

Growth inhibition of erlotinib on the resistant cells was different from that of the parent cell line; a reduced drug inhibition was observed in the resistant cells whereby a two-fold difference was recorded between the parent and the resistant cells. Prolonged incubation periods did not show differences in cell growth for all cells because the same trends were observed at 48 h, 72 h and 96 h. Erlotinib had the least effect on the erlotinib-resistant cells. However, the effects of
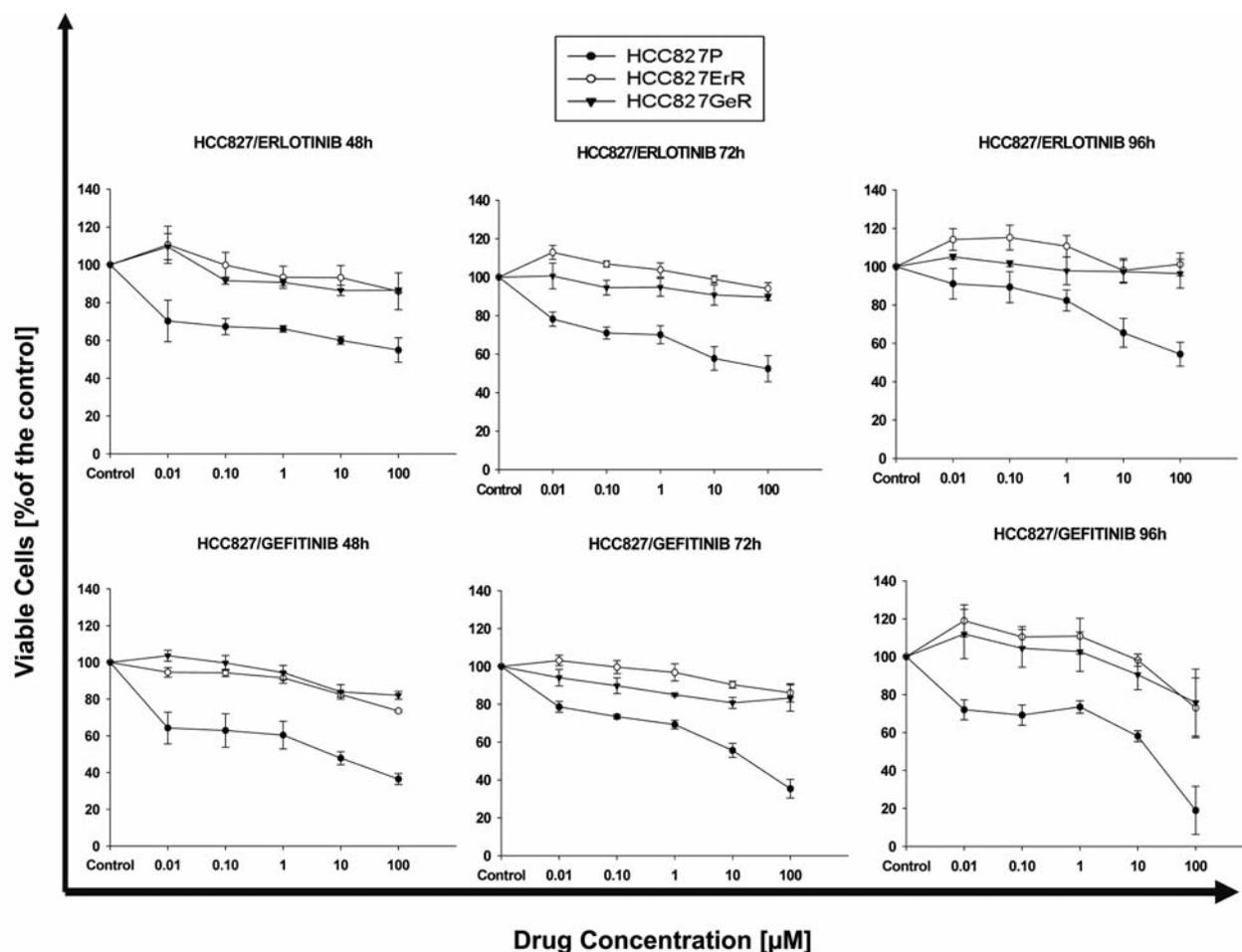
Figure 3. *Dose-response curves of HCC827 cells after developing resistance. Cell viability was assessed using the MTT assay and measurements were taken at 48 h, 72 h and 96 h after drug treatment. The resistant cells were run alongside the parent cells in order to compare resistant to sensitive cells.*

erlotinib on growth inhibition in the gefitinib-resistant cells were similar to that of the erlotinib-resistant cells.

Gefitinib also showed a dose-dependent inhibition in all the cells, with differences between the parent and the resistant cells. As was observed in erlotinib-treated cells, a drastic decrease in cell growth was also observed in HCC827P cells treated with gefitinib. This was recorded at 0.01 μM and increasing drug concentrations led to a gradual decrease in cell growth. This trend was observed at 48 h, 72 h as well as 96 h. Cell growth in HCC827ErR and HCC928Ger reduced gradually with increasing drug concentrations. However, the case was different at 96 h whereby cell growth increased at 0.01 μM but then decreased with increasing concentrations between 0.10 μM and 100 μM.

Growth inhibition of the drugs on the resistant cells reduced twofold when compared to the parent cells. Slight differences emerged between the incubation periods. At 48 h and 96 h, growth inhibition was slightly higher than at 72

h in HCC827P and the lowest growth was observed at 100μM at 96 h. Gefitinib showed the least effect on growth of erlotinib-resistant cells, however, the differences between the two resistant cell lines were not significant. Similar trends were observed in HCC827ErR and HCC827GeR cells treated with either erlotinib or gefitinib regardless of the drug cells were resistant to, and HCC827ErR cells showed the least sensitivity to the drugs.

Statistical analyses of the end-point measurements were carried-out and the results are presented in Figure 4.

The analyses were performed at 96 h because this is the point where maximum growth inhibition was reached for all the cells. On the one hand, samples at different concentrations were compared to those of untreated control to establish effects of the drugs on individual cell lines. On the other hand, the samples were compared against the parent cells for each drug concentration level to determine the differences in the effect of the drugs between the parent cells and the resistant cells.
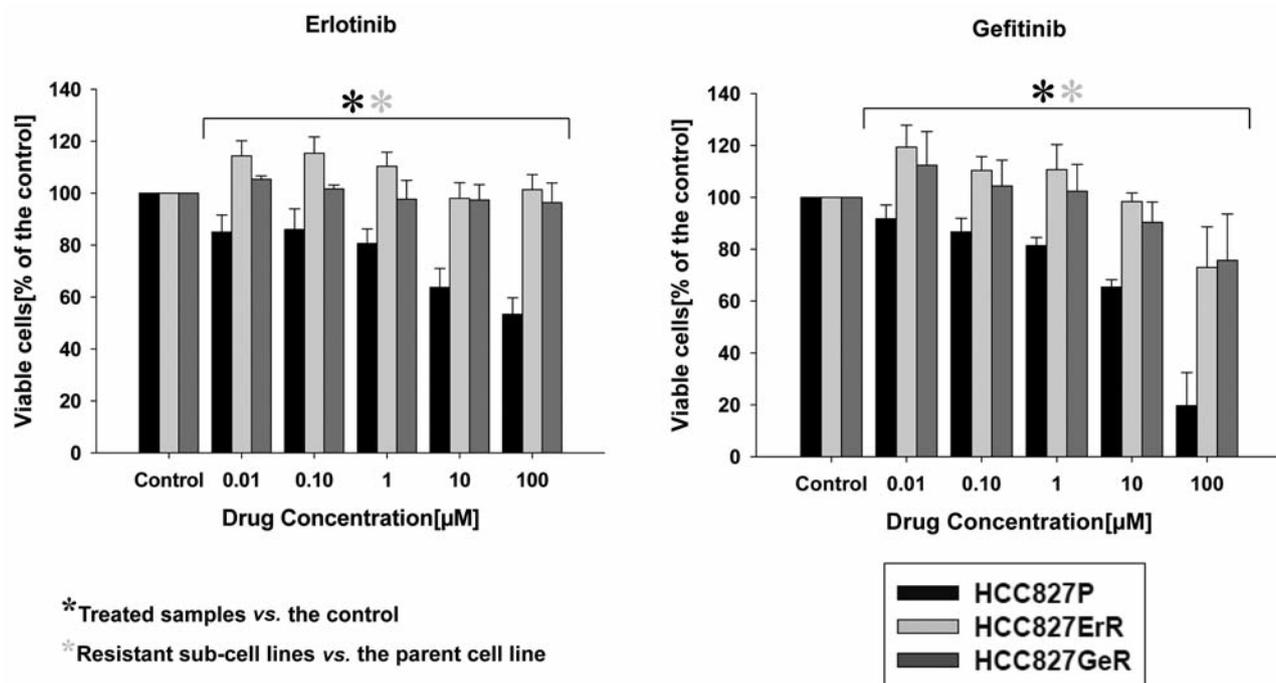
Figure 4. *Growth inhibition graphs of erlotinib and gefitinib at 96 h for HCC827P, HCC827ErR and HCC827GeR cells. Viability tests were carried out after cells were exposed to erlotinib and gefitinib for a period of three months.*

When erlotinib-treated cells were compared to the untreated control, significant differences were observed in HCC827P cells at all the concentrations ($p<0.05$) but not in HCC827ErR and HCC827GeR cells ($p>0.05$) with exceptions in HCC827ErR cells 0.01 μM ($p=0.007$) and 0.1 μM ($p=0.004$).

Gefitinib-treated cells were also compared to the untreated control, in this case HCC827P cells showed significant differences at all concentrations with exceptions of 0.01μM ($p=0.130$). No significant differences were observed in HCC827ErR and HCC827GeR cells at all concentrations ($p>0.05$). Another comparison was performed between the parent cells and resistant cells. In this case it was observed that there were significant differences between HCC827P and HCC827ER cells as well as between HCC828P and HCC827GeR cells at all concentrations ($p<0.05$).

The caspase 3/7 activity of lung cancer cells with primary and secondary resistance compared against sensitive cells. To find out if erlotinib and gefitinib induce apoptosis in lung cancer cells, the apoptosis assay was carried out and the results are presented in Figure 5. In comparison to controls, the activity of caspase 3/7 increased significantly in HCC827 cells ($p<0.001$) whereas in A549 ($p=0.705$) and H1299 ($p=0.582$) cells there was no effect observed on the caspase activity. The increase in drug concentration led to an increase

in caspase activity; however, this was only significant in HCC827 cells and not in A549 and H1299 cells. In HCC827 cells gefitinib showed an increase in the activity of the caspases compared to erlotinib in A549 and H1299 cells, however, there were no differences between the two drugs. Caspase activity of the treated samples was much higher in HCC827 cells than A549 and H1299 cell lines, the difference in caspase activity between HCC827 and the other cell lines was approximately three-fold.

The caspase 3/7 activity increased significantly in the HCC827P cells when compared to the untreated controls ($p<0.05$) in erlotinib-treated cells as well as gefitinib-treated cells. Significant differences were also observed between HCC827ErR cells, HCC827GeR cells and their corresponding untreated controls ($p<0.05$). The resistant cells (HCC827ErR and HCC827GeR) were compared against parent cells (HCC827P) and the results showed that caspase activity decreased in the resistant cells but the differences were not significant ($p>0.05$). Increasing drug concentration had no effect on the activity of caspase 3/7 for both erlotinib and gefitinib in all samples. Each drug had effects on both resistant cells since the effect on the caspase activity for both drugs was significantly reduced in both erlotinib- and gefitinib-resistant cells, *i.e.* erlotinib had the same effect on erlotinib and gefitinib-resistant cells and *vice versa*.
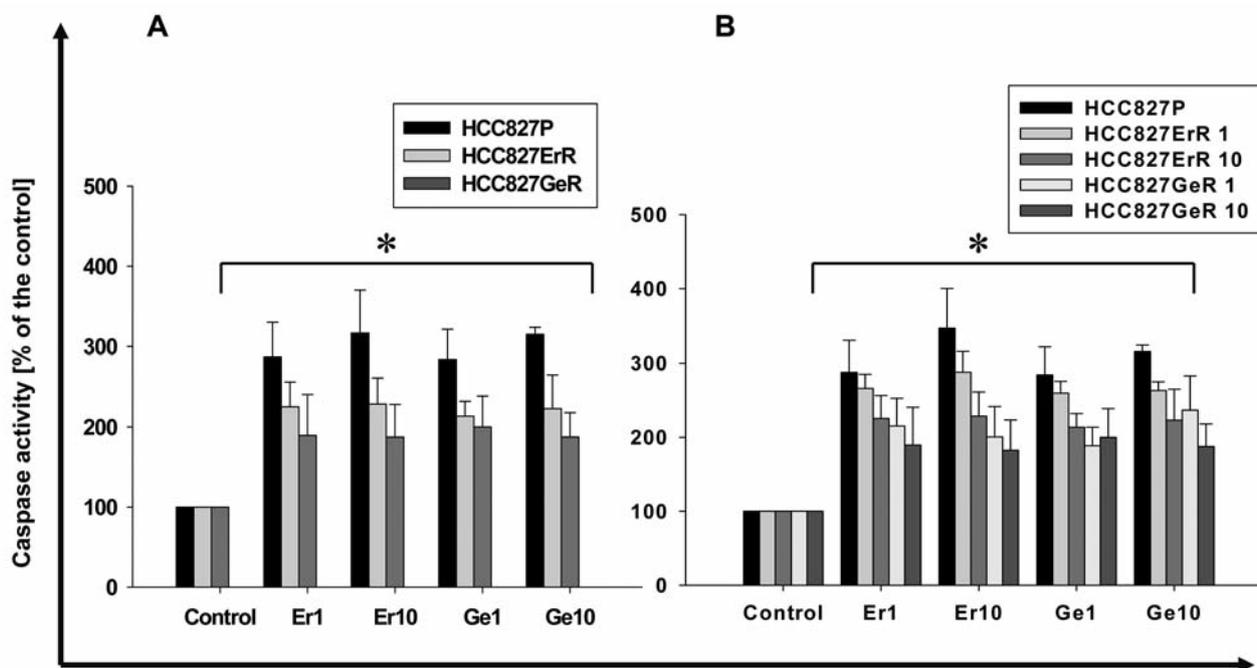
Figure 5. *The caspase activity of lung cancer cells was tested after exposing the cells to erlotinib and gefitinib (1 and 10 μM). A: The activity of caspase 3/7 of the individual cell lines was compared to the untreated control. B: The activity of caspase 3/7 of the resistant HCC827 cells was compared to the untreated control as well as to the parent cells (p<0.05). Er stands for erlotinib whereas Ge stands for gefitinib.*

Cell-cycle analysis of lung cancer cells with primary and secondary resistance compared to sensitive cells. Cell-cycle analysis was carried-out on all cell lines. The cells were grouped in 3 phases: apoptotic phase, G_0/G_1 and G_2/M. The assay was carried-out after exposing cells to erlotinib and gefitinib in order to determine the effect of the drugs on the cell cycle. The graphs in Figure 6 show the cell distribution in the various phases of the cell cycle.

A549 cells. Apoptotic cells numbers slightly increased following drug exposure in treated samples compared to the controls. Increased concentrations of erlotinib led to an increase of apoptotic cells. The number of apoptotic cells, however, decreased with increase in concentration of gefitinib. The majority of the cells (>50%) appeared in the G_1 phase although there was a shift towards the apoptotic phase in treated samples when compared to controls. The number of cells in the G_2/M phase remained constant in erlotinib-treated cells as well as in gefitinib-treated cells, however, differences were observed between the two drugs: gefitinib-treated cells were fewer in number in the G_2/M phase than erlotinib-treated ones, as well as controls. Statistically, apoptotic cells increased significantly when the treated samples were compared to the control ($p<0.05$). Moreover, no significant differences were observed in the G_0/G_1 phase

apart from in gefitinib-treated cells at 1 μM ($p=0.002$). The G_2/M phase did not show any significant differences in the number of cells when compared to the control.

H1299 cells. The cell distribution of the treated cells was compared to that of control cells of H1299 culture, there was a shift towards the apoptotic cells. These cells increased following exposure to erlotinib and gefitinib. Similarly to A549 cells, most of the cells (>50%) appeared in the G_0/G_1 phase. The number of cells in the G_2/M phase remained constant in all samples and the numbers were similar to those of controls. The increase in apoptotic cells was only significant in erlotinib-treated samples at 10 μM ($p<0.001$) when compared to controls. G_0/G_1 and G_2/M phases did not present significant differences in the cell numbers between treated samples and controls ($p>0.05$).

HCC827 cells. The cell distribution of HCC827 cells revealed a shift in all phases, *i.e.* apoptotic cells as well as cells in the G_0/G_1 phase increased in the treated samples but cell numbers in the G_2/M phase decreased. The majority (>50%) of the cells appeared in the G_0/G_1 phase in the treated samples but not in the controls, where G_0/G_1 and G_2/M showed equal numbers of cells. Apoptotic cells increased significantly in all treated samples compared to
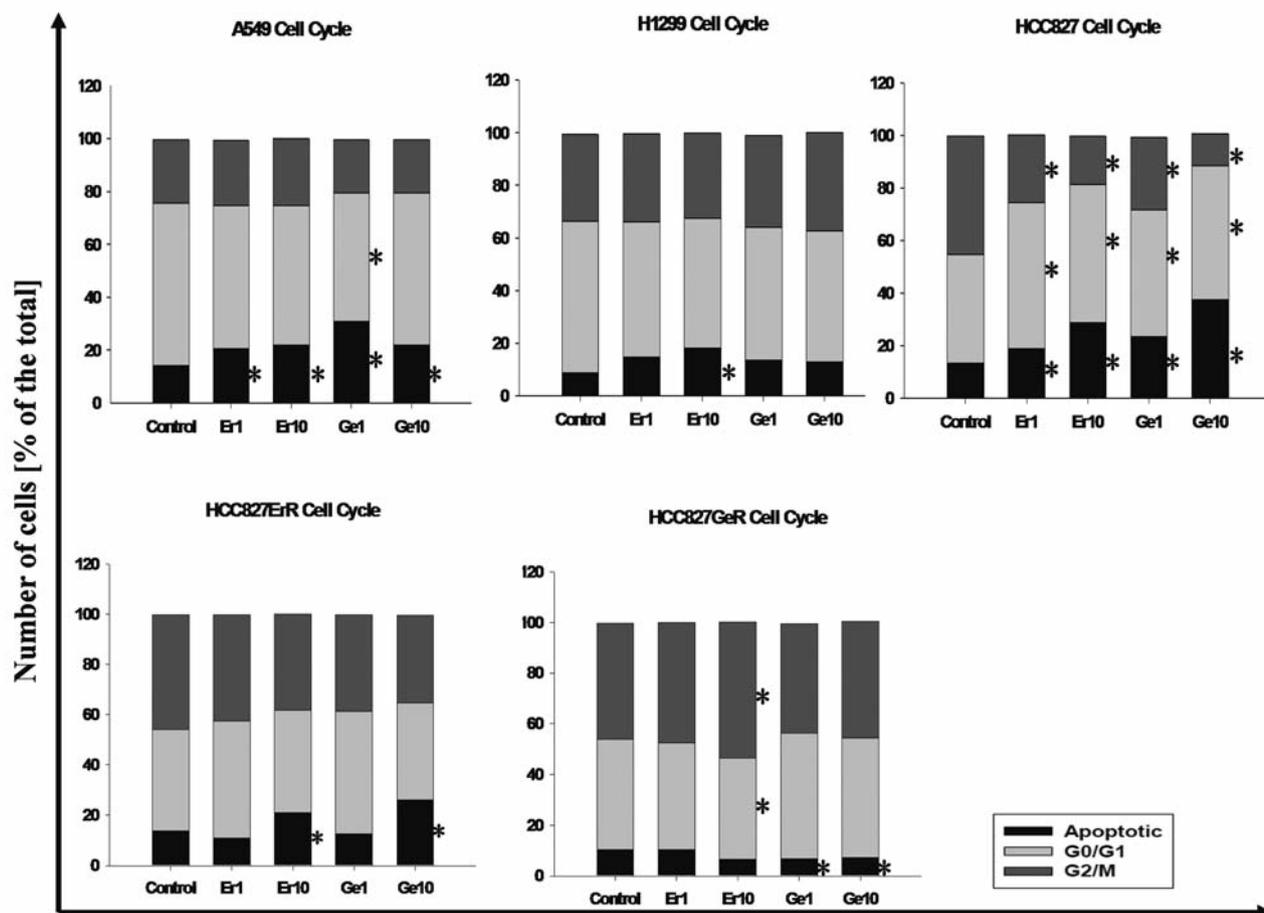
Figure 6. *The cell-cycle analysis of lung cancer cells was carried out 48h after exposing the cells to erlotinib and gefitinib at 1 and 10μM. The cells were stained with PI before being analyzed by FACS. The changes in the cell cycle patterns at G_0/G_1, G_2/M phases as well as in the apoptotic population were investigated (p<0.05). Er, Erlotinib; Ge, gefitinib.*

controls ($p<0.001$). Increase in drug concentration led to an increase of the apoptotic population in erlotinib-treated samples as well as gefitinib-treated samples. Significant differences were also observed in the number of cells in the G_0/G_1 and G_2/M phase when compared to controls ($p<0.001$). Unlike in A549 and H1299, HCC827 had more cells in the G_0/G_1 phase and these cells increased significantly in the gefitinib-treated samples at 10 μM.

A shift was observed in HCC827ErR cells towards the apoptotic population at 10 μM and towards G_0/G_1 phase at 1 μM; the majority of the cells (>50%) appeared in the G_0/G_1 phase. The number of cells in the G_2/M phase was similar in all the samples. Statistical analyses revealed that apoptotic cells increased significantly ($p<0.05$) in erlotinib-treated cells as well as gefitinib-treated cells at 10 μM. At a lower concentration (1 μM) of the drugs, apoptotic cells reduced when compared to the control but this was not significant. There were no significant differences in the number of cells

in the G_0/G_1 and G_2/M phases when treated samples were compared to the control.

In HCC827GeR cells, a shift was observed towards G_2/M in erlotinib-treated cells at 10 μM, and towards G_0/G_1 in gefitinib-treated cells at 1 μM as well as at 10 μM. There were similar number of cells in the G_0/G_1 and the G_2/M phases. Apoptotic cells reduced significantly ($p<0.001$) in gefitinib-treated cells but not in erlotinib-treated cells when compared to the control. There were no significant differences ($p>0.05$) observed in the G_0/G_1 and G_2/M phase in all samples apart from erlotinib-treated cells at 10 μM ($p<0.001$).

Discussion

Primary and secondary resistance to TKI in lung cancer were compared in the present study. Similarities and differences between cells with primary and secondary resistance were observed. From the viability tests it was evident that the cells

showed differences in sensitivity to the drugs, A549 was intermediate, H1299 resistant and HCC827 sensitive. Sensitivity to TKI has been reported to correlate to mutations within the EGFR (8, 15, 22). The tested cells had either a mutation in the *EGFR* (HCC827) or a wild-type *EGFR* (A549 and H1299). From the obtained results this study is in line with other studies which have reported sensitivity in cells with mutations and resistance in cells with a wild type EGFR. In this case H1299 and A549 represented primary resistant cells.

Development of secondary resistance was observed in HCC827 cells after exposing them to erlotinib and gefitinib for a period of three months. The viability results show that the resistant sub-cell lines had a reduced growth inhibition of the test substances as compared to the parent cells. Resistance development in patients undergoing treatment has been reported to occur within a period of 10 months (10, 11). Maemondo *et al*. and Margulescu *et al*. have reported progression-free survival in gefitinib-treated patients at a median of 10 months (23, 24). However in the cell culture model, resistance was observed as early as 3 months after the initial exposure of cells to the drugs.

Comparing the viability results of cells with primary resistant to those with secondary resistance; it was observed that the growth inhibition by the test substances on the cells was very low in both groups. The cell growth of the sensitive cells reduced after the cells developed resistance. This is an indication that erlotinib and gefitinib are unable to inhibit growth of both primary and secondary resistant cells although at different rates.

Besides cell growth inhibition TKI induces apoptosis in cancer cells and this was observed after treatment of TKI-sensitive cells with erolitinib and gefitinib. Apoptosis induction in TKI-sensitive cells has been reported (25). Erlotinib and gefitinib induced apoptosis in sensitive cells but not in those with primary resistance. The sensitive HCC827 line showed a three-fold increase, whereas A549 and H1299 did not show any differences in caspase activity. The resistant sub-cell lines on the other hand showed an increase in caspase activity, however this was lower than that of the parent cells. In contrast to A549 and H1299 cells the resistant sub-cell lines showed an increase in caspase activity. It can be concluded that despite HCC827 cells developing resistance, the drugs could still induce apoptosis although at a lower rate than in parent cells. Apoptosis assays results revealed that when cells develop resistance the ability of drugs to induce apoptosis decreases. However this is not comparable to cells with secondary resistance in which the drugs failed to induce apoptosis at all.

TKI have been reported to induce a G_0/G_1 cell-cycle arrest (26-29). In the present study this was only observed in the sensitive cells where the cell-cycle analysis revealed that the number of cells in the G_0/G_1 increased in the sensitive cells and not the resistant cells, when compared to controls of each individual cell line. The test substances, therefore, only induced cell-cycle arrest in the sensitive and not the resistant cells.

In general, a shift to the right was observed in HCC827 where by the apoptotic cells and cells in the G_0/G_1 phase increased, where as those in the G_2/M phase decreased. For the resistant cells the number of cells in all the phases did not show significant differences from the control apart from a few cases which are: A549 cells treated with gefitinib and erlotinib-resistant cells treated with erlotinib.

A G_0/G_1 cell-cycle arrest in the sensitive cells is evidence of EGFR inhibition by erlotinib and gefitinib. In order to proceed from G_0/G_1 phase to G_2/M phase, cells need sufficient supply of growth factors (30-32). Since the EFGR was blocked, the cells lacked supply of growth factors and did not proceed to the next phase. The opposite was observed for both primary and secondary resistant cells, since the cells were able to proceed to the G_2/M phase meaning that the EGFR was not inhibited and the cells had sufficient supply of growth factors.

It was evident that similarities existed between cells with primary and those with secondary resistance. This was observed through the viability assays as well as in the cell-cycle analysis. The apoptotic assays showed differences between primary resistance and secondary resistance. It would be interesting to further investigate the effects of TKI on cell signaling pathways to elucidate mechanisms of acquired resistance and to establish the differences between primary and secondary resistance.

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