Hsp90 inhibitor NVP-AUY922 enhances the radiation sensitivity of lung cancer cell lines with acquired resistance to EGFR-tyrosine kinase inhibitors

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Abstract. Acquired resistance to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) is a critical issue that needs to be overcome in the treatment of patients with non-small cell lung cancer (NSCLC) harboring *EGFR* activating mutations. EGFR and AKT are client proteins of the 90-kDa heat shock protein (Hsp90). Therefore, it was hypothesized that the use of Hsp90 inhibitors might allow the resistance to EGFR-TKIs to be overcome. Furthermore, Hsp90 inhibitors are known to function as radiosensitizers in various types of cancer. In the present study, we evaluated the radiosensitizing effect of the novel Hsp90 inhibitor, NVP-AUY922 (AUY), on NSCLC cell lines harboring *EGFR* activating mutations and showing acquired resistance to EGFR-TKIs via any of several mechanisms. We used HCC827 and PC-9, which are NSCLC cell lines harboring *EGFR* exon 19 deletions, and gefitinib-resistant sublines derived from the same cell lines with T790M mutation, *MET* amplification or stem-cell like properties. AUY was more effective against the gefitinib-resistant sublines with T790M mutation and *MET* amplification than against the parental cell lines, although the subline with stem cell-like properties showed more than a 10-fold higher resistance to AUY than the parental cell line. AUY exerted a significant radiosensitizing effect on the parental cell line and the *MET*-amplified subline through inducing G_2/M arrest and inhibition of non-homologous end joining (NHEJ). In contrast, the radiosensitizing effect of AUY was limited on the subline with stem cell-like properties, in which it did not induce G_2/M arrest or inhibition of NHEJ. In conclusion, combined inhibition of Hsp90 plus radiation was effective, and therefore a promising treatment alternative for overcoming major EGFR-TKI resistance, such as that induced by T790M mutation or *MET* amplification. However, other approaches are required to overcome minor resistance to EGFR-TKIs, such as that observed in cells with stem cell-like properties.

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Abbreviations: Hsp90, 90-kDa heat shock protein; AUY, NVP-AUY922; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; IR, ionizing radiation; IC_{10} and IC_{50}, 10% and 50% inhibitory concentrations; DNA DSB, DNA double-strand break

Key words: NVP-AUY922, 90-kDa heat shock protein inhibitor, epidermal growth factor receptor-tyrosine kinase inhibitor, drug resistance, radiation

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1). To improve the outcomes of patients with lung cancer, various novel therapeutic agents have been developed, including epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs). EGFR-TKIs show significant efficacy against non-small cell lung cancers (NSCLCs) harboring *EGFR* mutations, by inhibiting EGFR-AKT signaling (2-4). However, most of these tumors eventually acquire resistance to EGFR-TKIs (5,6). Several mechanisms of acquired resistance to EGFR-TKIs have been identified, such as secondary *EGFR* T790M (7) and minor mutations (8), and *MET* amplification (9). In addition, we also previously demonstrated an association between resistance to EGFR-TKIs and stem cell-like properties of the cells (10).

The 90-kDa heat shock protein (Hsp90) is a chaperone protein that modulates degradation, folding, and/or transport of a diverse set of critical cellular regulatory proteins (11). Critical oncogenic proteins, including receptor tyrosine kinases (RTKs) (e.g. EGFR) and their downstream proteins (e.g. AKT) are client proteins of Hsp90 (12,13), and mutated oncogenic proteins are more dependent on the functions of Hsp90 (14). Therefore, it was considered that Hsp90 may be a therapeutic target to overcome the resistance to EGFR-TKIs. Actually, Hsp90 inhibitors are effective against *EGFR*-mutated cell lines, even in those

that are resistant to EGFR-TKIs (15-17). Furthermore, Hsp90 inhibitors are known to exert a radiosensitizing effect through hypoxia-inducible factor-1α (HIF-1α), ataxia-telangiectasia mutated (ATM), checkpoint kinase 1 (CHK1), WEE1 G_2 checkpoint kinase (WEE1) (18-21) and other radioresistance-related client proteins. The radiosensitizing potential of Hsp90 inhibitors has been evaluated previously in NSCLC cell lines such as A549 and NCI-H460 (22,23). However, there are no reports focusing on the radiosensitizing effect of Hsp90 inhibitors on *EGFR*-mutated NSCLCs with acquired resistance to EGFR-TKIs.

In the present study, we evaluated the effect of the novel Hsp90 inhibitor NVP-AUY922 (AUY) in overcoming the major mechanisms of acquired resistance to EGFR-TKIs, such as *EGFR* T790M mutation and *MET* amplification, and the radiosensitizing effect of this compound. We also studied the radiosensitizing effect of AUY in overcoming acquired resistance induced by the acquisition of stem cell-like properties of the cells.

Materials and methods

Cell lines and reagents. *EGFR*-mutant cell lines HCC827 (exon19 del. E746-A750), and PC-9 (exon 19 del. E746-A750) were used. HCC827 was kindly gifted by Dr Adi F. Gazdar (The University of Texas Southwestern Medical Center, Dallas, TX, USA), who established this line with Dr John D. Minna (24,25). PC-9 was obtained from Immuno-Biological Laboratories (Takasaki, Gunma, Japan). Their gefitinib-resistant sublines, HCC827-GRmet with *MET* amplification, HCC827-GRstem with stem-cell like properties, and PC-9-GRt790m harboring the *EGFR* T790M mutation, were previously established by our group (10). All the cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), and grown in a humidified incubator with 5% CO_2 at 37˚C. AUY was obtained from Novartis Pharmaceuticals (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10 mM as a stock solution and stored at -20˚C until they were used for the *in vitro* experiments.

Cell proliferation assays. The proliferative ability of the cells was determined by a modified MTS assay using CellTiter 96® AQueous One solution reagent (Promega, Madison, WI, USA), as previously reported (26). The antiproliferative effects of AUY were determined based on the 10% and 50% inhibitory concentration (IC_{10} and IC_{50}), which denote the concentrations of AUY required to inhibit cell proliferation by 10% and 50%, respectively.

Clonogenic cell survival assays. Specified numbers of cells were seeded into each well in 6-well tissue culture plates, and after the cells became adherent (12 h), they were exposed to various concentrations of AUY, according to the obtained IC_{10} values, which were determined by cell proliferation assays. After a 24-h drug exposure, the plates were irradiated at 2, 4 or 6 Gy (ionizing radiation; IR), followed immediately by replacement of the culture medium with a drug-free conditioned medium. At 14 days after the IR, the colonies were fixed and stained using 0.4% crystal violet. The number of colonies containing at least 50 cells was counted. The survival data were fitted to a linear quadratic model as previously reported (23): $SF = \exp(-\alpha X - \beta X^2)$, where SF is the survival fraction, X is the radiation dose, and α and β are the fitted parameters. The results were evaluated using the surviving cell fractions at 2 Gy (SF2) and the radiation doses required for 10% survival (D_{10}), and the radiosensitizing effects of AUY were evaluated using the ratio of the D_{10} of the control cells to the D_{10} for each AUY concentration.

Table I. Inhibitory concentration values of NVP-AUY922.

Cell lines	Resistant mechanism	IC_{10} (nM)	IC_{50} (nM)
HCC827	-	2.5	21.0
HCC827-GRmet	*MET* amplification	2.7	7.0
HCC827-GRstem	Stem cell-like features	14.0	402.0
PC-9	-	2.8	9.1
PC-9-GRt790m	T790M mutation	2.9	6.7

IC_{10} and IC_{50}, 10% and 50% inhibitory concentration values.

Cell cycle analysis. The cell cycle distribution was evaluated by propidium iodide staining-based assay using the CycleTest™ Plus DNA reagent kit and FACSCalibur™ (both from Becton Dickinson, Franklin Lakes, NJ, USA). The cells were irradiated at 0 or 6 Gy (IR) after exposure or no exposure to 100 nM AUY for 24 h. At 48 h after IR, the cells were harvested and analyzed. Doublets, cell debris and fixation artifacts were gated out, and cell cycle analysis was performed using the software, CellQuest™, ver. 3.1.

Immunofluorescence staining for phosphorylated histone H2AX (γH2AX). DNA double-strand breaks (DNA DSBs) were evaluated by immunofluorescence staining for γH2AX (27). Each cell line was plated into chamber slides and after allowing the cells to become adherent (12 h), the medium was changed to that containing or not containing 100 nM of AUY. After a 24-h drug exposure, the plates were irradiated at 6 Gy, followed immediately by a change of the medium to a drug-free conditioned medium. The cells were fixed in 4% formalin for 15 min at 6, 24 and 48 h after the IR. Permeabilization and blocking were performed for 1 h using 10X PBS with 5% goat serum and 0.3% Triton X-100. Anti-γH2AX antibody at a 1:200 dilution was added as the primary antibody (Millipore, Billerica, MA, USA), followed by incubation overnight at 4˚C. Goat anti-mouse IgG conjugated Alexa Fluor® 555 (Life Technologies, Carlsbad, CA, USA) at a 1:1,000 dilution was added as the secondary antibody for 1 h and DAPI staining was performed using ProLong® Gold antifade reagent with DAPI (Life Technologies). The number of γH2AX foci in each nucleus was counted in at least 30 cells in each sample.

Statistical analysis. The Mann-Whitney U test was used to compare the data between the 2 groups. Data are expressed as the means ± standard deviations. Probability values (P) <0.05 were considered to indicate statistical significance. All the data

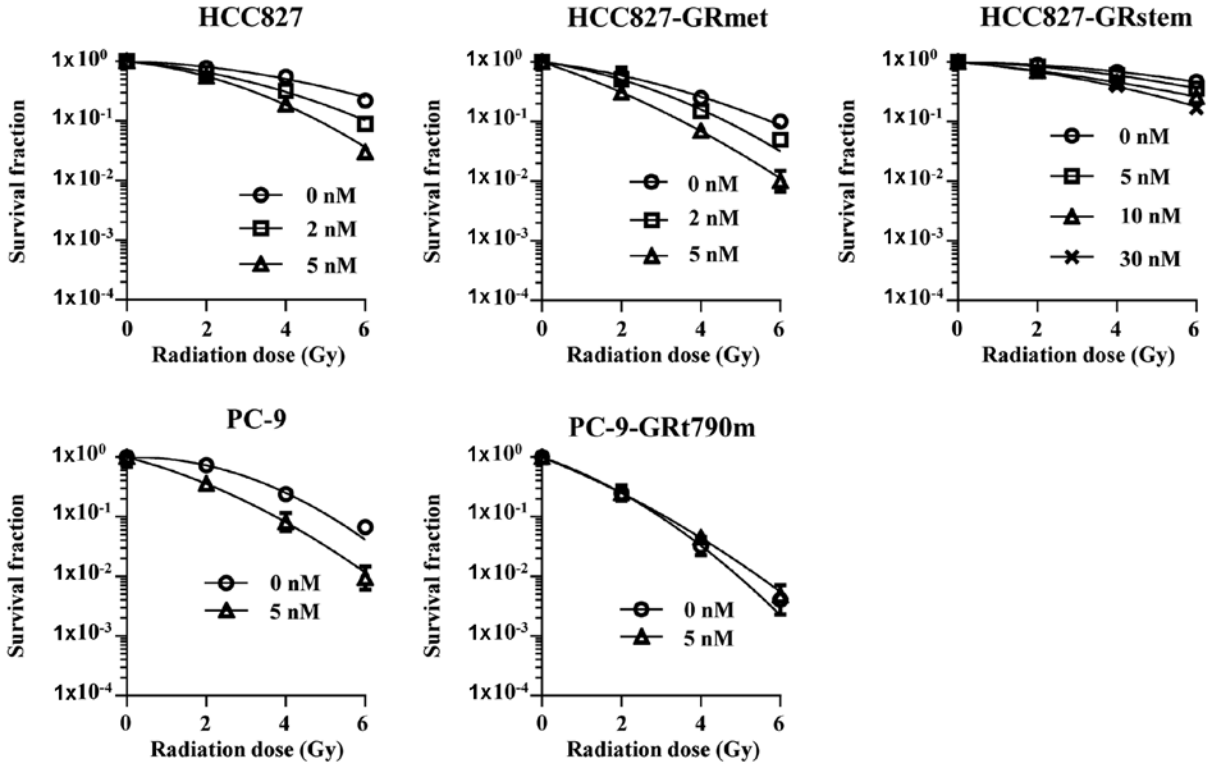

Figure 1. Survival curves of the clonogenic survival assays. Survival fractions indicate the ratio of the plating efficacy of the treated cells to the non-treated cells. Each symbol indicates the concentration of AUY. The cells were irradiated after exposure to AUY for 24 h and were plated into 6-well plates. The colonies were counted at 14 days after the IR. All experiments were performed at least three times and error bars indicate standard deviations.

were analyzed using GraphPad Prism, ver. 6.0.3, J (GraphPad Software, San Diego, CA, USA).

Results

AUY is effective for overcoming EGFR-TKI resistance in all of the cell lines examined, except for the cell line with stem cell-like properties. The IC_{10} and IC_{50} values of AUY in the parental cell lines and EGFR-TKI-resistant sublines are shown in Table I. HCC827-GRmet and PC-9-GRt790m cells were more sensitive to AUY than the parental cell lines. The IC_{50} value for HCC827-GRstem was over 20-fold as high as that for the other cell lines.

Radiosensitizing effect of AUY. The survival curves and parameters of the clonogenic cell survival assays are shown in Fig. 1 and Table II, respectively. The D_{10} values for HCC827-GRmet or PC-9-GRt790m cells were lower than those for the parental cell lines, while the value for HCC827-GRstem was higher than that for the parental cell line. Therefore, these sublines were more sensitive to IR than the parental cell lines, except for HCC827-GRstem. Radiosensitization was defined as a ratio of the D_{10} value for the control cells to that for the AUY-treated cells of >1.5. Our study revealed that both the parental cell line and HCC827-GRmet were radiosensitized, while HCC827-GRstem and PC-9-GRt790m were not radiosensitized by 5 nM of AUY.

G_2/M arrest is caused by IR with AUY. The proportions of HCC827 cells in the G_2/M phase following exposure to only IR and following exposure to both IR and AUY were 24 and 40%, respectively. The proportions of HCC827-GRmet cells in G_2/M phase following exposure to only IR and following exposure to both IR and AUY were 20 and 41%, respectively. In brief, exposure to both IR and AUY caused G_2/M arrest. However, in the case of the HCC827-GRstem cells, the proportion of cells in the G_2/M phase following exposure to only IR and following exposure to both IR and AUY were 20 and 22%, respectively. Therefore, HCC827-GRstem was resistant to G_2/M arrest even after combined IR plus AUY treatment (Fig. 2).

DNA repair ability of the EGFR-TKI-resistant cell lines. Repair of DNA DSBs was evaluated by determining the decrease in the number of γH2AX foci (Fig. 3). The numbers of γH2AX foci in the HCC827 cells after only IR were 47.6±21.4 and 32.7±28.8 at 6 and 48 h, respectively (P=0.01), while the corresponding values after exposure to both IR and AUY were 53.2±23.5 and 46.9±37.4 (P=0.33). The numbers of γH2AX foci in the HCC827-GRmet cell line after only IR were 48.2±24.5 and 37.3±24.5 at 6 and 48 h, respectively (P=0.02), and the corresponding values in the cells exposed to both IR and AUY were 48.2±22.7 and 57.8±23.1 (P=0.12). Furthermore, the numbers of γH2AX foci in the HCC827-GRstem cells exposed to IR alone were 45.4±23.2 and 16.4±8.7 at 6 and 48 h, respectively (P<0.01), and the corresponding values in the cells exposed to both IR and AUY were 49.1±27.1 and 15.9±9.7 (P<0.01). In the IR treatment group, HCC827 and HCC827-GRmet cells at 48 h showed a significant decrease in the numbers of γH2AX foci compared to those at 6 h. However,

Table II. Cloning efficiencies and radiosensitivity parameters.

Cell lines	Plating efficiency	SF2	α (Gy^{-1})	β (Gy^{-1})	D_{10} (Gy)[b]	D_{10} control/D_{10} + AUY922
HCC827	0.3±0.1	0.78±0.12	0.05±0.04	0.03±0.01	5	-
+2 nM AUY	0.3±0.1	0.65±0.04	0.13±0.01	0.04±0.004	3.6	1.4
+5 nM AUY	0.3±0.1	0.56±0.02	0.16±0.01	0.01±0.003	2.9	1.7
HCC827-GRmet	0.4±0.1	0.60±0.22	0.19±0.01	0.04±0.003	3.3	-
+2 nM AUY	0.4±0.02	0.51±0.04	0.22±0.03	0.06±0.01	2.6	1.2
+5 nM AUY	0.2±0.01	0.31±0.01	0.5±0.01	0.04±0.004	1.7	1.9
HCC827-GRstem	0.5±0.1	0.90±0.25	0.02±0.05	0.02±0.01	6.9	-
+5 nM AUY	0.5±0.1	0.84±0.17	0.04±0.02	0.02±0.01	5.9	1.2
+10 nM AUY	0.6±0.04	0.71±0.08	0.14±0.01	0.01±0.005	4.8	1.4
+30 nM AUY	0.5±0.04	0.68±0.02	0.14±0.01	0.02±0.003	4.2	1.7
PC-9	0.85±0.27	0.74±0.08	0.03±0.1	0.09±0.04	3.4	-
+5 nM AUY	0.06±0.01	0.36±0.09	0.4±0.1	0.06±0.05	2	1.8
PC-9-GRt790m	0.4±0.1	0.25±0.09	0.5±0.4	0.08±0.17	1.5	-
+5 nM AUY	0.2±0.02	0.25±0.02	0.6±0.2	0.04±0.07	1.5	1.0

AUY, NVP-AUY922; SF2, surviving cell fractions at 2 Gy; D_{10}, radiation doses required for 10% survival; D_{10} control/D_{10} + AUY922, ratio of D_{10} of control to D_{10} for each AUY concentration. Data are shown as the mean ± standard deviation from at least 3 experiments.

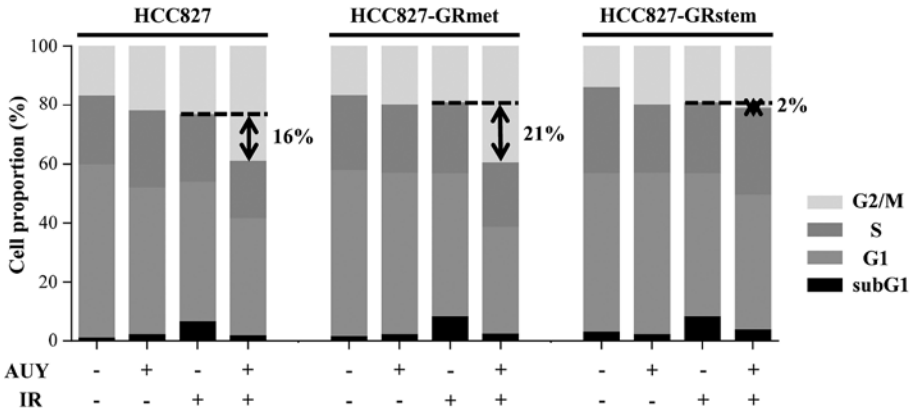

Figure 2. Effects of the combined treatment if IR and AUY on cell cycle distribution. The cells were irradiated at 6 Gy after exposure to 100 nM of AUY for 24 h, and were then harvested and analyzed at 48 h after the IR. The cell cycle distribution was determined by flow cytometry after propidium iodide staining. AUY, NVP-AUY922; IR, ionizing radiation.

in the IR plus AUY group, the significant difference was not achieved between the numbers of γH2AX at 6 h and those at 48 h. In contrast HCC827-GRstem cells showed a significant decrease in the number of γH2AX foci after both IR treatment alone and after combined IR plus AUY treatment.

Discussion

In the present study, AUY was effective against EGFR-TKI-resistant cells with secondary mutation of *EGFR* or other RTK dependence. These results are concordant with previous reports (15-17). We demonstrated that combined exposure to IR and AUY caused G_2/M arrest and inhibition of DNA DSB repair, and radiosensitized EGFR-TKI-resistant cell lines with major resistance mechanisms such as T790M mutation and *MET* amplification. However, the DNA repair ability of the EGFR-TKI-resistant cell line with stem cell-like properties was maintained even after combined treatment with IR and AUY, and the radiosensitizing effect of AUY on this cell line was limited.

Notably, the cell lines with acquired resistance to EGFR-TKIs associated with T790M mutation or *MET* amplification were more sensitive to IR than the parental cell lines in our study. Das and colleagues showed that NSCLC with activating *EGFR* mutations were sensitive to IR (28). They proposed two possible mechanisms to explain this finding. i) Elevated or aberrant signaling from the mutant EGFR may override the IR-induced checkpoint. ii) Translocated EGFR binds to the promoter region of DNA-dependent protein kinase (DNA-PK) (29), while mutated EGFR may not be able to bind to it. Although the precise reasons for the greater radiosensitivity of the EGFR-TKI-resistant-sublines than that of the parental

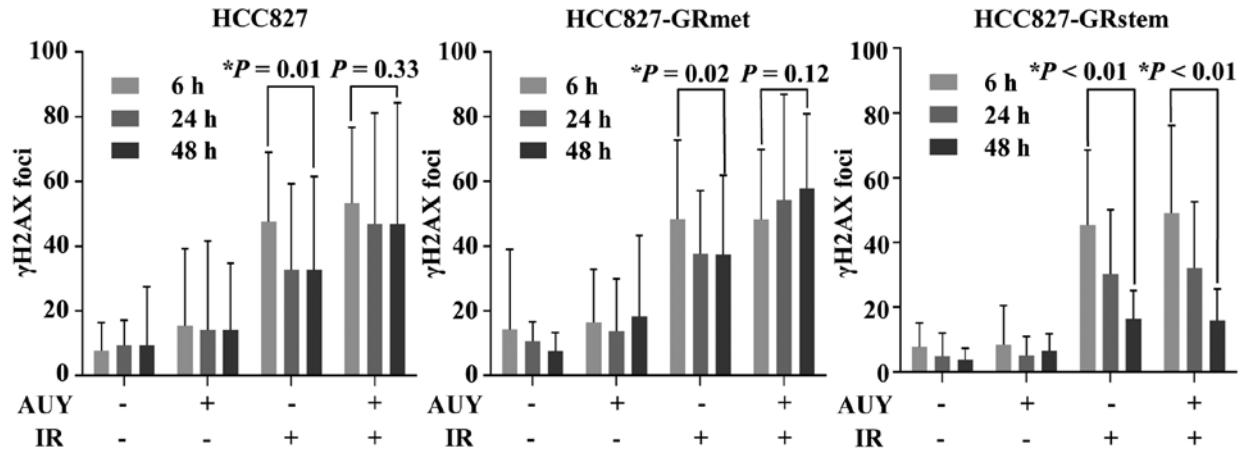

Figure 3. DNA DSB repair after IR. The numbers of γH2AX foci in each nucleus reflecting DNA DSBs are shown. The cells were irradiated at 6 Gy after exposure to 100 nM of AUY for 24 h, and stained by immunofluorescence staining; the number of γH2AX foci in the cells was then counted. Each symbol indicates the time point after IR. Error bars indicate the standard deviations. The numbers of γH2AX foci at 6 h after IR were compared to the numbers at 48 h after IR in each of the cells using the Mann-Whitney U test. *P<0.05 (two-tailed). DSB, double strand breaks; AUY, NVP-AUY922; IR, ionizing radiation.

cell lines in our cohort could not be clearly elucidated, the acquired resistance mechanisms may have an influence. The signaling from amplified-*MET* may also override the IR-induced checkpoint, or secondary *EGFR* mutations such as the T790M mutation may also affect the binding of EGFR to the DNA-PK promoter.

Previously, several mechanisms to explain the radiosensitizing effect of Hsp90 inhibitors have been reported (18-21). IR causes G_2/M arrest through the ATM-CHK pathway (30). As ATM and CHK are client proteins of Hsp90, Hsp90 inhibitors enhance the G_2/M arrest caused by IR (23,31-33). Alternatively, Hsp90 inhibitors impair non-homologous end joining (NHEJ) through DNA-PK/ATM (23,31,33-35). These phenomena were also shown in our cohort, except in the HCC827-GRstem cell line.

Cancer stem cells show activation of DNA DSB repair by NHEJ through the DNA-PK/ATM-CHK pathway, and several cancers, including NSCLCs, show radioresistance (35-38). HCC827-GRstem, an EGFR-TKI-resistant cell line with stem cell-like properties, also showed radioresistance and activation of DNA DSB repair. Therefore, it was expected that the Hsp90 inhibitor might allow the radioresistance of this cell line to be overcome, since DNA-PK, ATM and CHK are client proteins of Hsp90. However, combined treatment with IR and AUY of the HCC827-GRstem cell line produced neither G_2/M arrest nor inhibition of DNA DSB repair.

As secondary mutations of *EGFR* or other RTK dependence accounts for EGFR-TKI resistance in over 60% of cases (39,40), these can be defined as the major resistance mechanisms. Combined therapy with IR and AUY is a promising option to overcome these major EGFR-TKI resistances; on the other hand, other minor resistance mechanisms, such as those in cells with stem cell-like properties, require other approaches.

In conclusion, combined therapy with IR and AUY is effective to overcome major acquired resistance to EGFR-TKIs such as that associated with the T790M mutation or *MET* amplification, while the effect on resistance associated with stem cell-like properties of the cells was limited. Further investigation is warranted to elucidate the mechanism of acquired resistance to EGFR-TKIs associated with stem cell-like properties of cells.

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