Thermal cycling hyperthermia sensitizes non-small cell lung cancer A549 cells to EGFR-tyrosine kinase inhibitor Erlotinib

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Abstract

Molecular-targeted therapy has emerged as a mainstream treatment for non-small cell lung cancer (NSCLC), the most common lung cancer, which has been the leading cause of cancer death for both men and women. Erlotinib (Erl), a targeted therapy drug which triggers epidermal growth factor receptor (EGFR) pathways, has been proved to have noticeable response rate for NSCLC cells. However, it has achieved only limited treatment effect, due to intrinsic and acquired resistance among most NSCLC patients. Therefore, chemosensitizers are required to potentiate the efficacy of Erl in NSCLC treatment. The study proposed a novel thermal therapy, thermal cycling hyperthermia (TC-HT), as a supplement to amplify the effect of Erl, and proved that it can sensitize A549 NSCLC cells to Erl via the downstream of EGFR signaling cascades. In addition, we found that TC-HT not only greatly enhanced the anticancer effect of Erl but also remarkably reduced the half-maximal inhibitory concentration (IC50) to as little as 0.5 μM. Besides, via regulation of thermal dosage, TC-HT alone can produce excellent antineoplastic effect without hurting the normal cells. The method is expected to be applicable to other combination therapies and may be a starter for more sophisticated, side-effect-free anticancer treatments.

Introduction

As one of the most common type of cancer, lung cancer was the largest source of cancer-related deaths in 2020, according to World Health Organization (WHO). In particular, Non-small cell lung cancer (NSCLC) accounted for 85% of lung cancer deaths and there has yet to be effective treatment for it [1]. Existing therapies for NSCLC treatment include surgery, chemotherapy, radiation, and targeted therapy. As chemotherapy alone is not effective, due to low response rate, there is urgent need for combination treatment or new agents targeting one or more molecular pathways.

Targeted treatment, meant to inhibit cancer growth via focus on critical molecules, has been regarded as a promising therapy to treat NSCLC patients [2]. Among various targeted therapies, anti-epidermal growth factor receptor (EGFR) has been the one with most extensive application in clinical treatment of NSCLC [3]. EGFR is a receptor tyrosine kinase of the ErbB family which plays a key role in cell proliferation, survival, differentiation, and migration [4]. When activated by ligand binding, EGFR will autophosphorylate and trigger downstream signalling cascades, including MAPK and PI3K-Akt pathways [5], leading to cell cycle progression and inhibiting pro-apoptotic proteins. Therefore, several EGFR-inhibiting agents, such as monoclonal antibodies and tyrosine kinase inhibitors (TKIs) have been developed for application in anticancer treatments [6]. Erlotinib (Erl), a first generation EGFR-TKI, has exhibited significantly better response rate and benefits in NSCLC patients than conventional platinum-based chemotherapy [7], capable of prolonging the survival of patients with advanced NSCLC after chemotherapy. Regrettably, most tumors will develop acquired resistance to Erl over time [8], plus such side effects as rash and diarrhea for long-term administration of high-dose Erl [9]. Therefore, it is worthwhile to evaluate the effect of combination treatment in potentiating the efficacy of Erl at

low dose, in place of chemotherapy drugs, such as cisplatin and paclitaxel, which have been applied as sensitizers to enhance the efficacy of Erl [10, 11], with such side effects as hypertension and severe diarrhea, though [12].

In this work, we proposed a novel thermal therapy, thermal cycling hyperthermia (TC-HT), as substitute for chemotherapy drug or agent, to study whether such physical cue, TC-HT, may be an effective sensitizer to amplify the anticancer effect of Erl. Given the safety and effectiveness of TC-HT reported in our previous studies [12, 13], we aimed to probe the effect of combining Erl and TC-HT in A549 NSCLC cell line. The results showed that TC-HT significantly enhanced the anticancer effect of Erl by sensitizing A549 cells to Erl. As a consequence, the Erl dosage required to achieve significant anticancer effect was greatly reduced. The approach is expected to solve the problems of low response rate and acquired resistance for cancer drugs in NSCLC patients, while reducing side effects significantly. The enhanced anticancer effect was achieved in part via EGFR and downstream pathways as shown by western blot analysis and flow cytometry. The results show that Erl and TC-HT synergistically weaken the phosphorylation of EGFR and modulate the p-JNK and Akt protein expressions, thereby inducing the mitochondria-dependent apoptosis. Besides, TC-HT alone, to our surprise, can exhibit potent anticancer effect by raising the temperature setting slightly, which is comparable to the in vitro efficacy of chemotherapy drugs. This result was not observed in pancreatic cancer PANC-1 cells. Moreover, normal lung cell line IMR-90 was almost unharmed with the same TC-HT treatment condition.

The study demonstrated for the first time that TC-HT can potentiate the anticancer efficacy of Erl on A549 cells, with the potential for application in other chemotherapy or targeted therapy drugs, thereby reducing their needed dosage in

treatment. Moreover, by tuning its parameters according to cell types, TC-HT could hinder growth of some cancer cells, without drug use. These findings suggest that combination TC-HT and Erl is a promising approach in anticancer treatment, which sheds light on combining TC-HT with other anticancer drugs for potential treatment.

Materials and methods

Cell culture

The human NSCLC cell line A549 was purchased from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan). A549 cells were seeded on cell culture flask and cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO_2 at 37˚C.

Drug treatment and TC-HT exposure

Erl, purchased from MedChemExpress (MCE), was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck KGaA) to a concentration of 5 mM as the stock solution and stored at -20˚C. A549 cells were seeded in 96-well plates overnight before treated with various concentrations of Erl. The TC-HT groups were exposed to a thermal cycling treatment with a high temperature period for 3 min and a cooling period for 30 sec, and this protocol was repeated for different number of cycles as shown in Fig 1A. The actual temperatures sensed by the cells in the bottom of the well were measured by a needle thermocouple. For the combination treatment, TC-HT was applied for 1 h before Erl treatment. After the single or combination treatment, the cells

were maintained in the cell culture incubator for an additional 72 h for further analyses.

Cell viability assay

The viability of A549 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich; Merck KGaA) assay. After drug or TC-HT treatment, the medium was replaced by DMEM containing 0.5 mg/mL MTT, and incubated for 4 h at 37˚C. The formazan crystals were dissolved by 10% sodium dodecyl sulfate (SDS) (Bioman Scientific Co., Ltd.) overnight. Then, the optical density of formazan solution was measured at 570 nm by an ELISA microplate reader. Background absorbance at 690 nm was subtracted, and the final absorbance value was expressed as a percentage of the untreated controls to represent the cell viability.

Western blot analysis

After treatment with Erl or TC-HT or in combination, cells were washed with ice-cold PBS and lysed in RIPA lysis buffer (EMD Millipore) on ice for 30 min. Cell lysates were clarified by centrifugation at 23,000 × g for 30 min at 4˚C, and then the protein concentration in the supernatant fraction was measured by the Bradford protein assay (Bioshop). Proteins (20–40 μg) were subjected to 10% SDS-PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore). After incubation in a blocking buffer (5% bovine serum albumin/TBST) for 1 h at room temperature, the membranes were immunoblotted with diluted primary antibodies at 4˚C overnight. The specific primary antibodies against p-EGFR, PARP, p-JNK, p-Akt, t-Akt, MTH1 (Cell Signaling Technology, Inc.), and GAPDH (GeneTex, Inc.) were used. After being washed with TBST, the membranes were incubated with horseradish

peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Images were visualized with an enhanced chemiluminescence substrate (Advansta, Inc.) and quantified by Amersham Imager 600 imaging system (AI600, GE Healthcare Life Sciences).

Mitochondrial membrane potential (MMP) measurement

After treatment with Erl or TC-HT or in combination, cells were washed and resuspended with phosphate buffered saline (PBS) followed by staining with 20 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Enzo Life Sciences International Inc.) for 30 min at 37˚C in the dark. The fraction of mitochondrial depolarization in cells is indicated by the decrease of fluorescence intensity measured by a flow cytometer (FACSVerse; BD Biosciences).

Statistical analysis

The statistical results were expressed as the average from three independent experiments and the error bars are ± standard deviation. Statistical analyses were performed by using OriginPro 2015 software (OriginLab). Differences of statistical significance were determined by one-way analysis of variance followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TC-HT for in vitro application

The thermal cycling treatment was applied with a modified PCR machine as described previously [13, 14]. The schematic temperature and duration settings are shown in Fig 1A, where the temperature was increased to the desired high temperature setting and

maintained for 3 min, followed by a natural cooling period for 30 sec. In practice, the heating device was switched off in the cooling process, and the low temperature setting was chosen to be 37˚C to mimic human body temperature. A single cycle containing a high temperature and a low temperature period was repeated for 10 times in this study. Fig 1B showed the actual temperatures in the culture well measured by a thermocouple at two different high temperature settings. As can be seen in Fig 1B, the cycling temperature comes to an equilibrium state after the third heating period, thereafter the temperature cycling between 41.5~43˚C for moderate temperature TC-HT and 42.5~45.6 ˚C for high temperature TC-HT, respectively. In this work, we focus to study the synergistic anticancer effect of Erl and TC-HT in A549 cancer cells, so we mainly adopted the moderate temperature TC-HT treatment (41.5~43˚C) in the following experiments.

Fig 1. In vitro-applied TC-HT. (A) Schematic representation of the high and low temperatures and their duration settings during TC-HT treatment. (B) Actual temperatures with two different high temperature settings in the culture well measured every 15 sec by a needle thermocouple.

TC-HT potentiates the anticancer efficacy of Erl in A549 NSCLC cells

To determine the effect of combination Erl and TC-HT treatment on human NSCLC cell viability, A549 cells were treated with different concentrations of Erl with or without TC-HT for 72 h, followed by cell viability assessment by MTT assay. Since the purpose of this study was to investigate the synergistic effect of Erl and TC-HT, the dose of Erl was chosen to below 10 μM, which is relatively low in the in vitro experiment. As shown in Fig 2A, Erl exhibited antineoplastic effect in a

concentration-dependent manner. The half-maximal inhibitory concentration (IC50) of Erl was about 10 μM in A549 cells after 72 h treatment. In contrast to treatment with Erl alone, TC-HT therapy sensitizes A549 cells to Erl and synergistically reduces the cell viability to about 30% of the control group (Fig 2A). It is noteworthy that the combination of Erl and TC-HT had synergistic activities as compared with Erl monotherapy, which remarkably reduced the IC50 to as little as 0.5 μM (up to 20-fold decrease). To verify the effect of this combined treatment in normal cells, the normal lung cell line IMR-90 was used and applied with the same treatment. As shown in Fig 2B, the cell viability of IMR-90 normal lung cell line was almost unaffected by the Erl and TC-HT regardless of the treatment procedure. The result implies that TC-HT is not only a riskless sensitizer to natural compounds [13, 14], but also an effective and safe sensitizer to increase the anticancer effect of targeted therapy drug.

Fig 2. Effect of Erl or TC-HT or in combination on the cell viability. (A) MTT viability assay of A549 NSCLC cells treated with different concentrations of Erl or in combination with moderate temperature TC-HT treatment (41.5~43˚C). (B) MTT viability assay of IMR-90 normal lung cells treated with 5 and 10 μM Erl or in combination with moderate temperature TC-HT treatment (41.5~43˚C). Data represent the mean ± standard deviation (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test (***$P < 0.001$).

Effect of Erl combined with TC-HT on EGFR protein expression in A549 cells

The next step was examination of signalling pathways involved in the anticancer mechanism of Erl and TC-HT combination. With NSCLC cells exhibiting high EGFR expression [15], targeted therapy drugs were applied to inhibit the expression. Meant

specifically for cancers with EGFR mutation, Erl inhibits cancer cells by blocking the intracellular phosphorylation of tyrosine kinase with such mutation [7]. However, NSCLC may develop acquired resistance to Erl over time, according to some studies [8]. In the study, as shown in Fig 3, Erl alone caused a slight decrease in EGFR phosphorylation compared to the control cells. Moreover, the study finds that while TC-HT alone can significantly inhibit p-EGFR expression, the combination of Erl and TC-HT can induce more pronounced inhibition effect on p-EGFR expression. It suggests that TC-HT may modulate the resistance of A549 cells to Erl and alter the protein expression of EGFR downstream pathways.

Fig 3. Effect of Erl combined with TC-HT on p-EGFR protein expression in A549 cells. Western blot analysis of p-EGFR protein expression in A549 cells treated with 10 μM Erl, moderate temperature TC-HT, and the combination treatment. The expression levels of p-EGFR were normalized to GAPDH. Each relative expression level was compared with control and represented as fold relative to control. Data represent the mean ± standard deviation (n=3). Differences of statistical significance were determined by one-way ANOVA followed by Tukey's post-hoc test (**$P < 0.01$ and *$P < 0.05$).

Effect of Erl combined with TC-HT on EGFR signalling pathways in A549 cells

In the study, we then examined the expressions of JNK and Akt proteins [16] in A549 cells. JNK protein, a EGFR downstream protein belonging to the MAPK family, is deemed to be responsive to stress stimuli and heat shock, with its phosphorylation capable of altering the activities of many proteins that reside in mitochondria or act in the nucleus and trigger apoptosis [17]. As shown in Fig 4A, TC-HT treatment

significantly increased the expression of p-JNK protein, compared with the control or Erl-treated A549 cells. The result shows that TC-HT alone can effectively promote apoptosis. Furthermore, we find that the treatment combining Erl and TC-HT is much more effective in upregulating the p-JNK expression than TC-HT alone, as it can boost apoptosis even more. On the other hand, another EGFR downstream protein Akt is involved in cellular survival pathways by inhibiting apoptotic process [18]. Once activated by phosphorylation, Akt actively regulates a number of transcription factors facilitating the expression of survival proteins. Fig 4B shows that the expression of phosphorylated Akt (p-Akt) in Erl-treated A549 cells is lower than the untreated cells, while the inhibitive effect on phosphorylation of Akt increases further in TC-HT treatment and is even higher in the combination Erl and TC-HT treatment. Meanwhile, total Akt (t-Akt) protein exhibits a trend similar to that of p-Akt protein, suggesting that decrease in the phosphorylation of Akt in these treatments is caused by the reduction in the amount of Akt, leading to a weakened survival signal. Moreover, it is known that both JNK and Akt signalling pathways are important regulators in influencing mitochondrial function. The injured mitochondria releases cytochrome-c into the cytoplasm, cleaving caspase 9 and thus activating caspase 3 in the downstream [19], which enters further the nucleus and cleaved PARP. It should be noted that PARP plays an important role in mitochondria-mediated apoptosis, in addition to being a key enzyme for DNA repair. In apoptosis, PARP protein is typically cleaved and inactivated, thereby suppressing DNA repair and causing programmed cell death [20].

To understand the apoptosis mechanism in A549 cells triggered by the combination Erl and TC-HT treatment, the study evaluated the expression of apoptosis-related proteins with western blot analysis. Fig 4C shows that the ratio of

cleaved PARP to full length PARP in TC-HT treated cells was much higher than that in Erl-treated cells. Moreover, we find that the ratio of cleaved PARP to full length PARP in combination Erl and TC-HT treatment was much stronger than that in TC-HT treatment alone, indicating the combination treatment enhances apoptosis of A549 cells via mitochondrial pathway. In the same vein, MTH1 protein has received increasing attention in cancer treatment, due to its key role in DNA repair [21]. High expression of MTH1 is deemed to be a sign for the metastasis of NSCLC [22]. Therefore, the study examined MTH1 protein expression via western blot analysis. As shown in Fig 4D, MTH1 expression was found to decrease in Erl-treated cells, while the inhibitive effect on MTH1 protein expression was much higher in TC-HT treatment, which increased further in the combination Erl and TC-HT treatment. The result reveals that the combination of Erl and TC-HT may prevent the MTH1-related DNA repair and further trigger apoptosis.

Fig 4. Effect of Erl combined with TC-HT treatment on survival- and apoptosis-related protein expressions in A549 cells. The anticancer experiments were conducted in A549 cancer cells treated with 10 μM Erl, moderate temperature TC-HT, and the combination treatment. Western blot analysis of p-JNK (A), p-Akt and t-Akt (B), cleaved PARP and full length PARP (C), MTH1 (D) protein expressions. The expression levels of p-JNK, p-Akt, t-Akt, and MTH1 were normalized to GAPDH and cleaved PARP was normalized to full length PARP. Each relative expression level was compared with control and represented as fold relative to control. Data represent the mean ± standard deviation (n=3). Differences of statistical significance were determined by one-way ANOVA followed by Tukey's post-hoc test (***$P < 0.001$ and **$P < 0.01$).

Effect of combination Erl and TC-HT treatment on MMP in A549 cells

The mitochondria-dependent apoptosis is associated with a loss in MMP, causing the release of cytochrome *c* and other apoptotic factors [23]. To confirm the apoptosis caused by Erl and TC-HT could be related to mitochondrial disruption, the MMP was assessed using $DiOC_6(3)$ fluorescence staining by flow cytometric analysis. As shown in Fig 5, single treatment by Erl or TC-HT did not significantly alter the MMP level in comparison with the untreated control. However, the combination Erl and TC-HT treatment markedly increased the MMP depolarization in A549 cells, indicating that Erl- and TC-HT-induced apoptosis was mediated by mitochondrial dysfunction.

Fig 5. Flow cytometric analysis of MMP in A549 cells treated with Erl and/or TC-HT. A549 cells were treated with 10 μM Erl, moderate temperature TC-HT, and the combination of both treatments. (A) MMP was evaluated by the $DiOC_6(3)$ fluorescent dye and detected by flow cytometer. (B) Quantification of the cells with decreased MMP was measured. Data represent the mean ± standard deviation (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test (**$P < 0.01$)

Discussion

The study demonstrated the synergistic anticancer effect of the combination of Erl and TC-HT on A549 NSCLC cells, with TC-HT significantly potentiating the efficacy of targeted therapy drug Erl in inhibiting the phosphorylation of EGFR and modulating the JNK and Akt downstream pathways. EGFR is a receptor tyrosine kinase critical for the initiation and development of malignant tumors via MAPK and

PI3K/Akt pathways. Although the EGFR-TKIs such as Erl are meant to target and inhibit the EGFR pathways, their clinical benefit has been limited, due to the intrinsic and acquired resistance to TKIs among most NSCLC patients. Amid the effort to combat the resistance of EGFR-TKIs [24], combination treatment has been deemed the most promising approach to overcome the resistance problem [25]. Several studies have probed the effect of combining Erl and other anticancer agents such as cisplatin, monoclonal antibodies, aspirin, and capsaicin [10, 26-28], whose applications, however, have been hampered by unpredictable drug interactions or side effects so far. The study demonstrated for the first time the effect of the physical stimulation of TC-HT in boosting the anticancer effect of Erl, without hurting the normal lung cells, thereby sidestepping drug interaction problem and significantly reducing the needed dosage of Erl, which lessens side effects.

In this work, we find that TC-HT can synergize with Erl significantly to combat the A549 cancer cells and also reduce the IC50 of Erl remarkably. To prove that low-dose Erl in synergy with TC-HT can induce cell apoptosis, the western blot analysis of PARP protein was performed in A549 cells treated with 2 μM Erl, TC-HT, and the combination of both treatments. Given cleavage of PARP as the hallmark of apoptosis, the ratio of cleaved PARP to full length PARP was also evaluated in the low-dosage treatment experiment. Fig 6 shows that low-dosage Erl does not change the ratio of cleaved PARP to full length PARP, which, though, increases significantly with the application of TC-HT alone. The increase scale is even higher with combination of low-dosage Erl and TC-HT, underscoring intensification of apoptosis signal, in agreement with MTT assay.

Fig 6. Effect of low-dosage Erl combined with TC-HT on PARP protein

expression in A549 cells. Western blot analysis of PARP protein expression in A549 cells treated with 2 μM Erl, moderate temperature TC-HT, and the combination of both treatments. The expression level of cleaved PARP was normalized to full length PARP. Each relative expression level was compared with control and represented as fold relative to control. Data represent the mean ± standard deviation (n=3). Differences of statistical significance were determined by one-way ANOVA followed by Tukey's post-hoc test (**$P < 0.01$ and *$P < 0.05$).

Hyperthermia has long been a promising cancer treatment method, to be applied alone or in combination with other conventional therapies [29]. The treatment, however, often causes cell damage, due to over dosage, a problem which can now be overcome by TC-HT, thanks to its ability to control thermal dosage, as its intermittent cooling process can prevent excessive thermal dosage accumulation and subsequent cytotoxic cell damage. Excessive thermal dosage may induce mitochondrial damage and oxidative stress not only in cancer cells but also in normal cells [30]. However, studies have shown that cancer cells have higher ROS levels and thus they are more sensitive to thermal stress [31]. In this study, the results show that moderate temperature TC-HT treatment (41.5~43˚C) alone inhibited the cell viability of A549 NSCLC cells slightly, reducing their viability to 80% of the control group, without harming IMR-90 normal lung cells. To further examine the effect of TC-HT alone on cancer cells at higher temperature, the temperature setting in TC-HT application was raised to the range of high temperature TC-HT treatment (42.5~45.6 ˚C), which resulted in significant inhibition of the cell viability of A549 NSCLC cells, with their viability dropping to only 17.9% of that of the control group (Fig 7A). Moreover, the treatment did not have much influence on the viability of IMR-90 normal lung cells,

which remained at 80% of that of the control group, even in the case of combining the treatment with 10 μM Erl (Fig 7B). The results suggest that TC-HT is a promising treatment method for NSCLC and perhaps other cancer cells, capable of reducing the reliance on drugs.

Fig 7. Effect of high temperature TC-HT or Erl or in combination on the cell viability. (A) MTT viability assay of A549 NSCLC cells treated with different concentrations of Erl or in combination with high temperature TC-HT treatment (42.5~45.6 ˚C). (B) MTT viability assay of IMR-90 normal lung cells treated with various concentrations of Erl or in combination with high temperature TC-HT treatment (42.5~45.6 ˚C). Data represent the mean ± standard deviation (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test (***$P < 0.001$).

In conclusion, the study demonstrated for the first time that TC-HT, a novel thermal treatment, sensitizes A549 cells to EGFR-TKI Erl, via the downstream of EGFR signalling cascades, including the MAPK and PI3K-Akt pathways. The study raised the potential for application of TC-HT in other combination therapy, thereby reducing drug dosage and side effects significantly. Furthermore, TC-HT may become a drug-free cancer treatment method via precision regulation of thermal dosage according to cell types in its application. Further studies are in urgent need to examine specific TC-HT parameters in treating different kinds of cancers.

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Competing Interests

The authors have declared that no competing interests exist.

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Writing – review & editing: Chih-Yu Chao.

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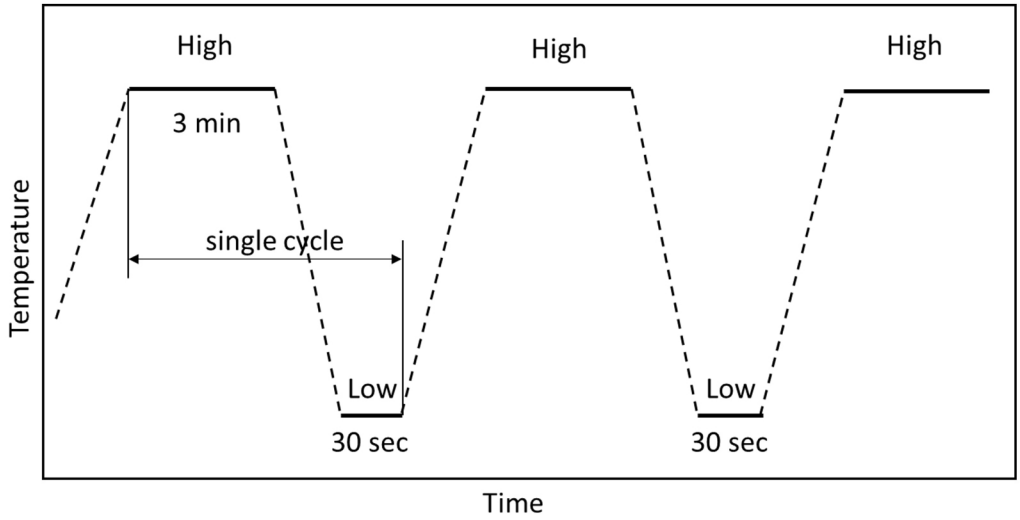
Figure 1

A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single cycle
Temperature
Low
Low
30 sec
30 sec
Time

B

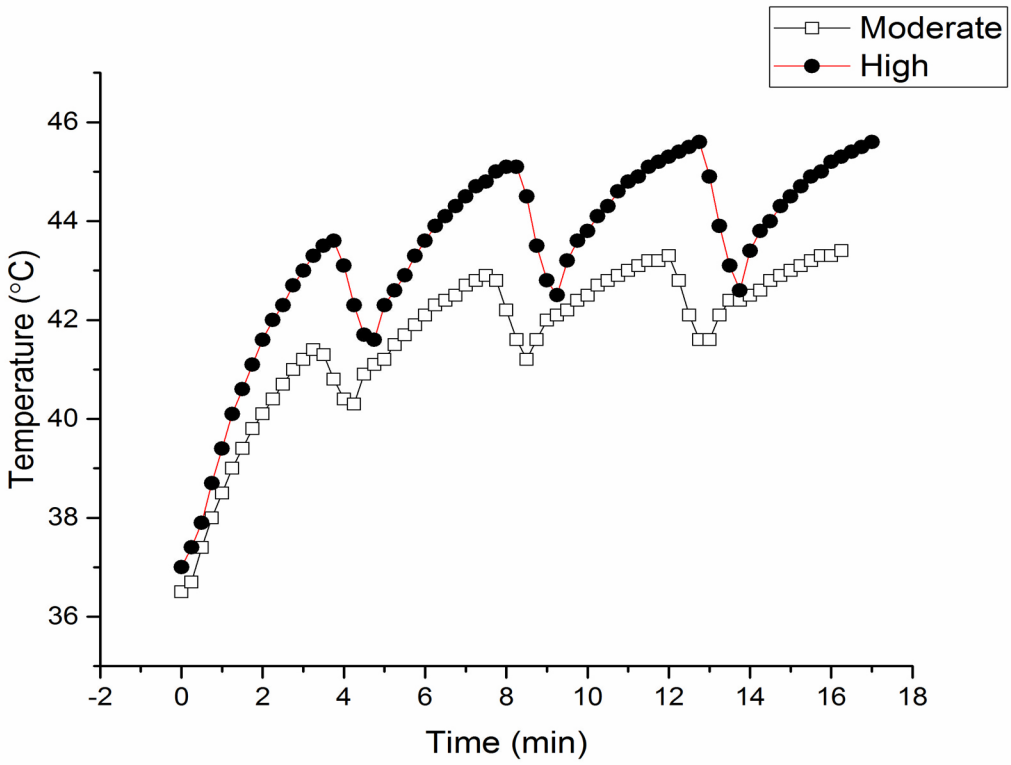

Moderate
High
Temperature (°C)
46
44
42
40
38
36
-2
0
2
4
6
8
10
12
14
16
18
Time (min)

Figure 2

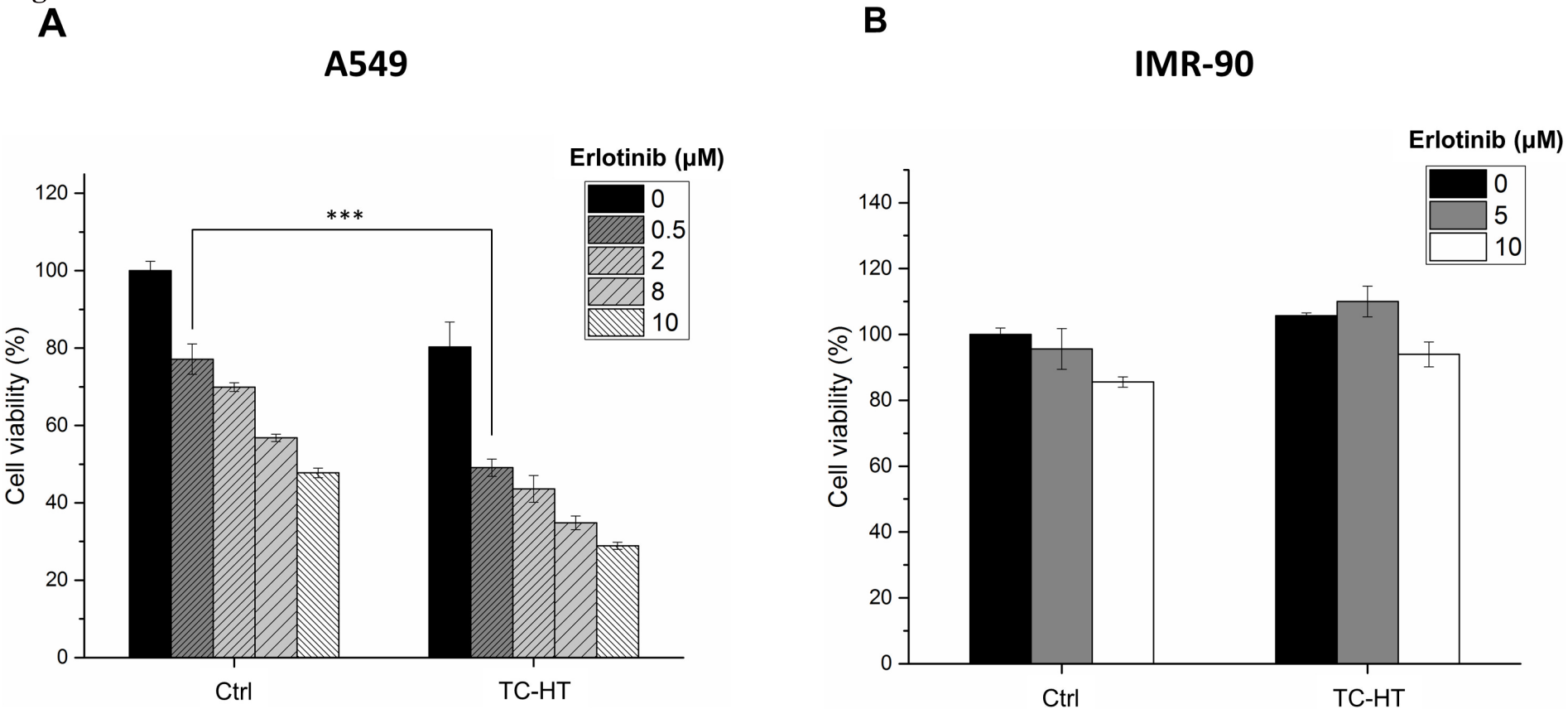

A
A549
Erlotinib (μM)
0
0.5
2
8
10
***
Cell viability (%)
0
20
40
60
80
100
120
Ctrl
TC-HT
B
IMR-90
Erlotinib (μM)
0
5
10
Cell viability (%)
0
20
40
60
80
100
120
140
Ctrl
TC-HT

Figure 3

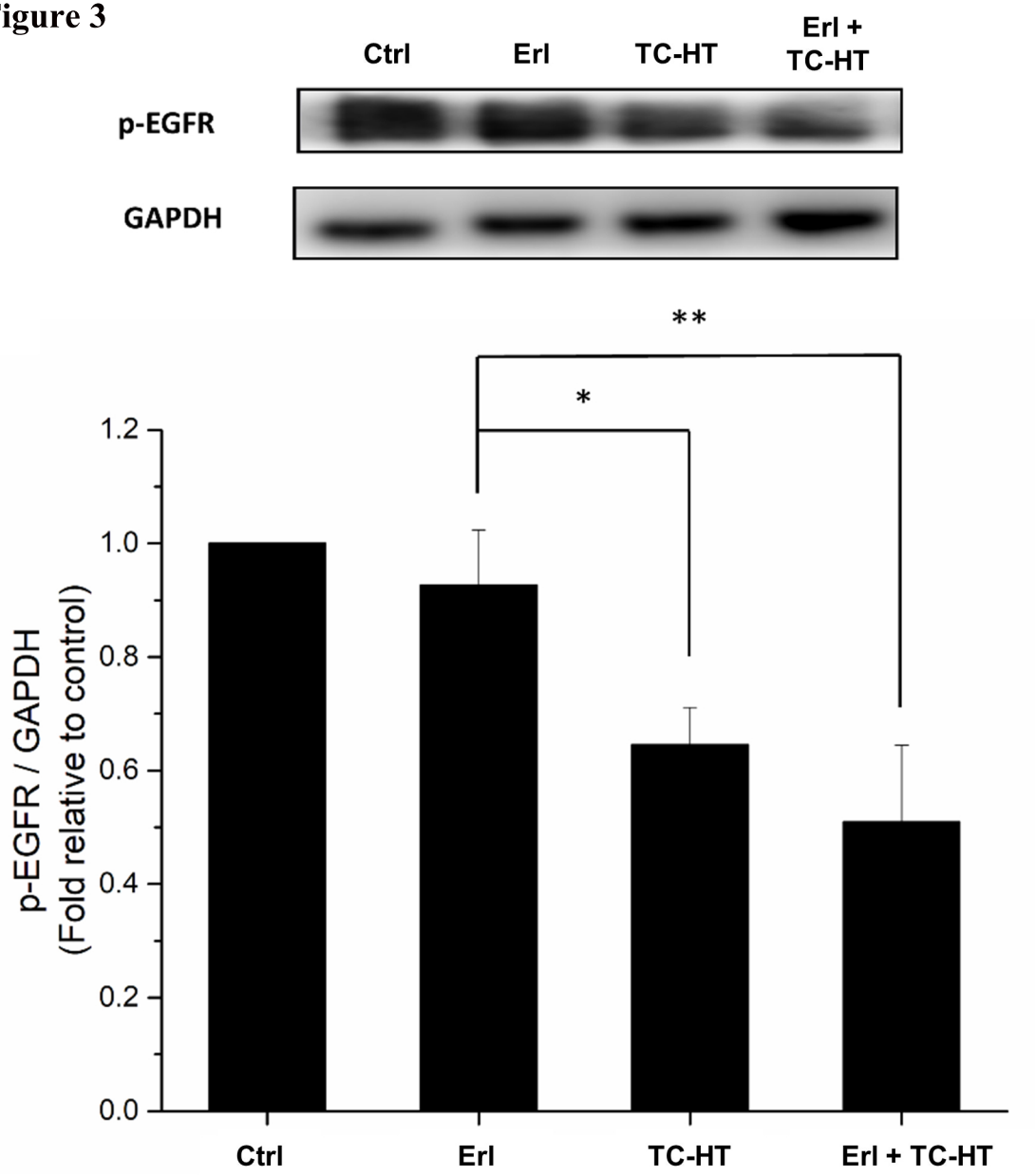

Ctrl
Erl
TC-HT
Erl + TC-HT
p-EGFR
GAPDH
**
*
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p-EGFR / GAPDH
(Fold relative to control)
Ctrl
Erl
TC-HT
Erl + TC-HT

Figure 4

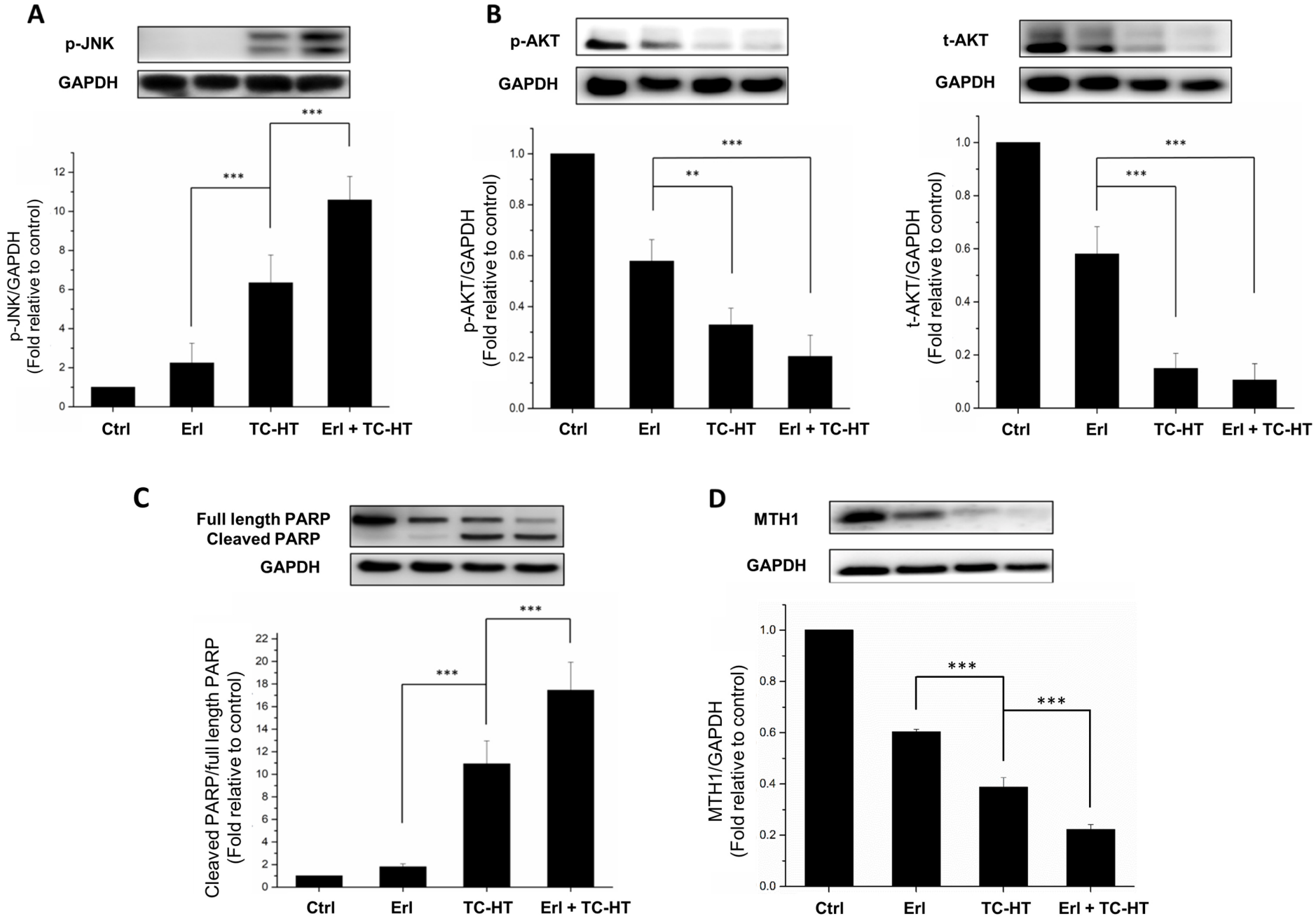

A
p-JNK
GAPDH
p-JNK/GAPDH
(Fold relative to control)
***
***
Ctrl
Erl
TC-HT
Erl + TC-HT
B
p-AKT
GAPDH
p-AKT/GAPDH
(Fold relative to control)
***
**
t-AKT
GAPDH
t-AKT/GAPDH
(Fold relative to control)
***
***
C
Full length PARP
Cleaved PARP
GAPDH
Cleaved PARP/full length PARP
(Fold relative to control)
***
***
D
MTH1
GAPDH
MTH1/GAPDH
(Fold relative to control)
***
***

Figure 5

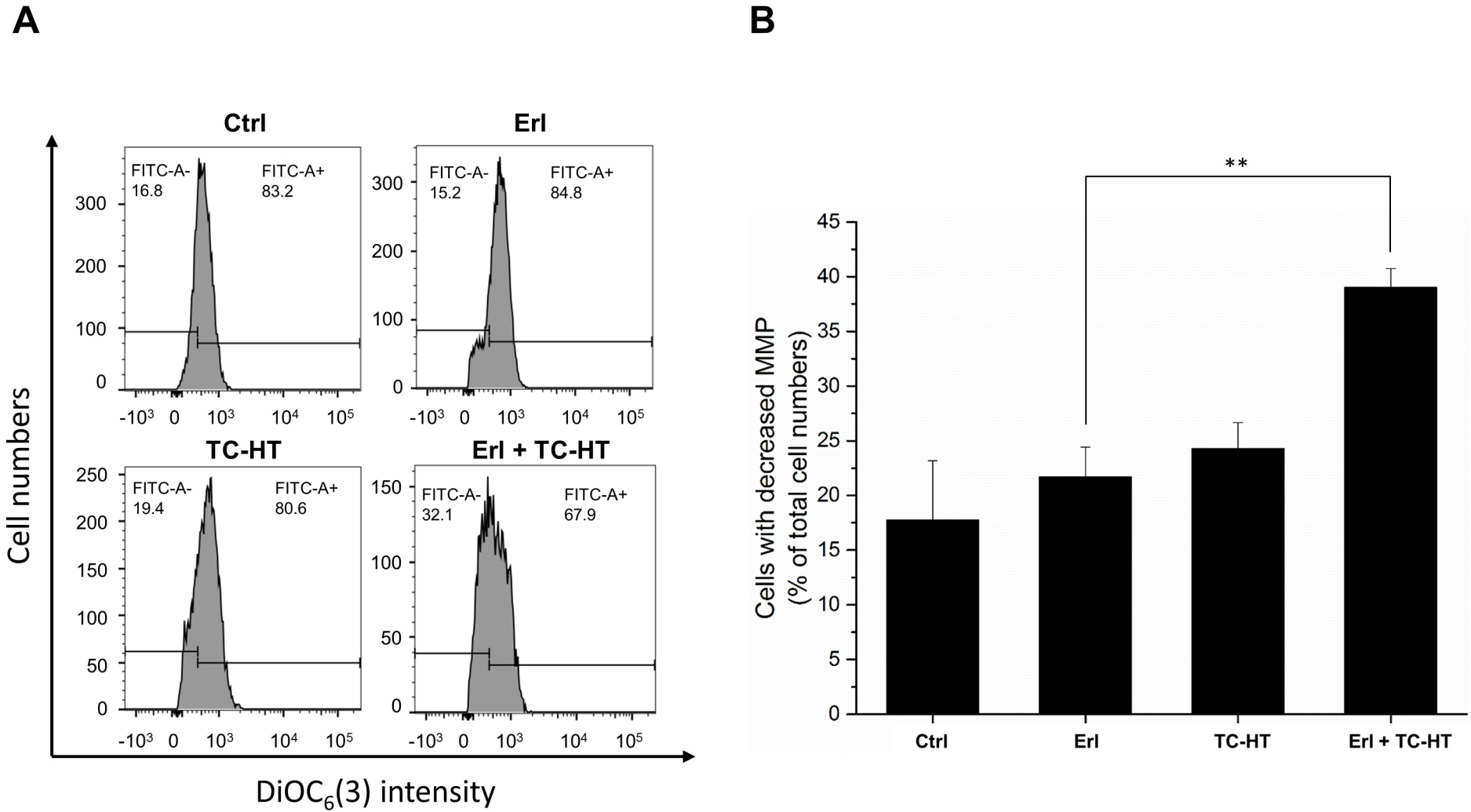

A
Ctrl
FITC-A-
16.8
FITC-A+
83.2
Erl
FITC-A-
15.2
FITC-A+
84.8
TC-HT
FITC-A-
19.4
FITC-A+
80.6
Erl + TC-HT
FITC-A-
32.1
FITC-A+
67.9
Cell numbers
DiOC6(3) intensity
B
**
Cells with decreased MMP
(% of total cell numbers)
Ctrl
Erl
TC-HT
Erl + TC-HT

Figure 6

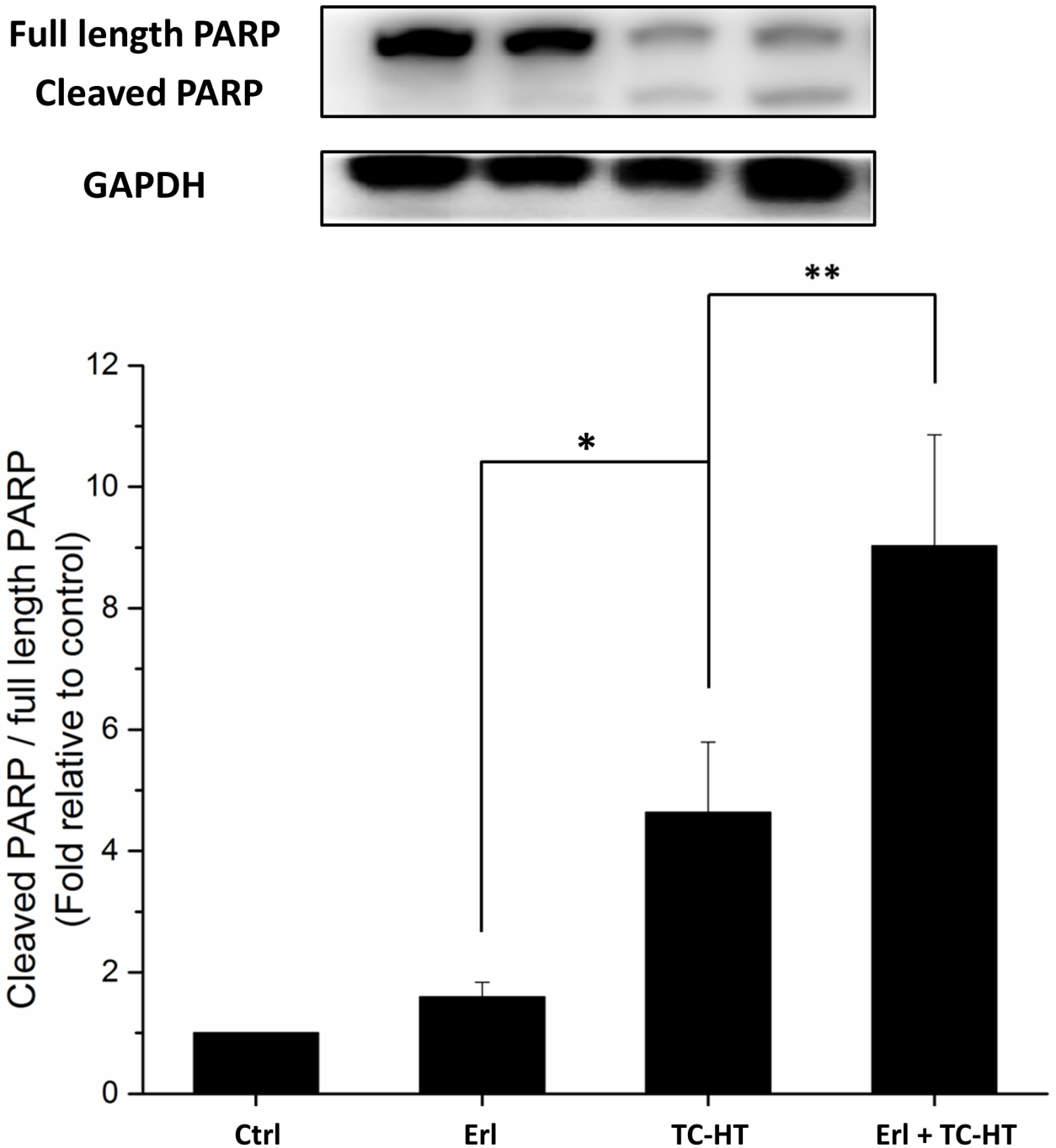
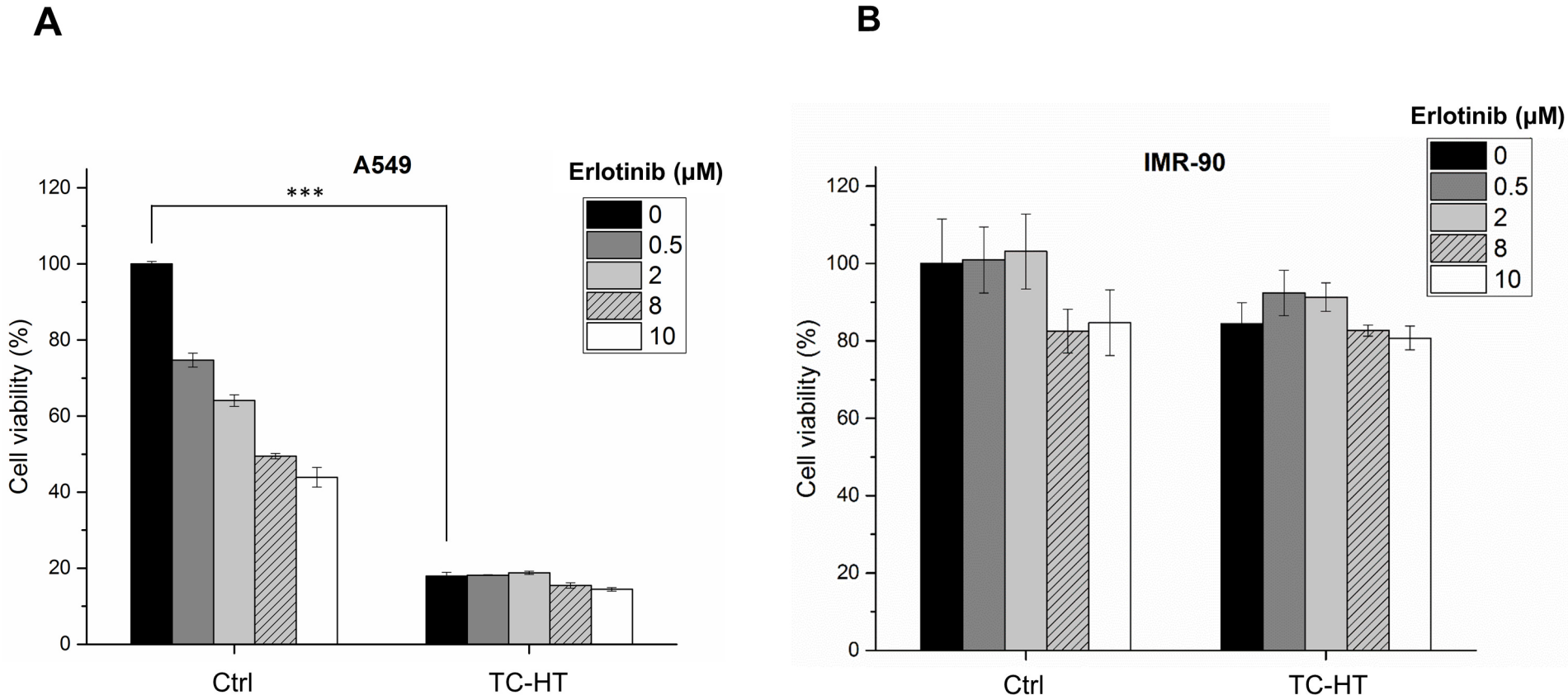

Full length PARP
Cleaved PARP
GAPDH
**
*
Cleaved PARP / full length PARP
(Fold relative to control)
0
2
4
6
8
10
12
Ctrl
Erl
TC-HT
Erl + TC-HT

Figure 7


A
A549
Erlotinib (μM)
0
0.5
2
8
10
***
Cell viability (%)
0
20
40
60
80
100
120
Ctrl
TC-HT
B
IMR-90
Erlotinib (μM)
0
0.5
2
8
10
Cell viability (%)
0
20
40
60
80
100
120
Ctrl
TC-HT