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Design, synthesis and biological evaluation of novel heptamethine cyanine dye-erlotinib conjugates as antitumor agents

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ABSTRACT

Epidermal growth factor receptor tyrosine kinase (EGFR-TK) has been proved as a target for the treatment of non-small cell lung cancer (NSCLC) with specific gene mutations. However, EGFR-TK inhibitors (EGFR-TKIs) need to enter cancer cells and then competitively interact with the active site of tyrosine kinase receptors to suppress the downstream signaling pathway to inhibit tumor proliferation. In this study, in order to improve the tumor cell targeting ability of EGFR-TKI, EGFR-TKI erlotinib was conjugated with the cancer cell-targeting heptamethine cyanine dyes to form seventeen novel erlotinib-dye conjugates. The efficiency of tumor targeting properties of conjugates against cancer cell growth and EGFR-TK inhibition was evaluated *in vitro*. The result revealed that most erlotinib-dye conjugates exhibited stronger inhibitory effect on A549, H460, H1299 and MDA-MB-231 cell lines than the parent drug erlotinib. Meanwhile, representative compounds exhibited weak cytotoxicity on human normal mammary epithelial MCF-10A cells. Moreover, the conjugate **CE17** also showed ~14-fold higher EGFR-TK inhibition activity (IC_{50} = 0.124 μM) than erlotinib (IC_{50} = 5.182 μM) in A549 cell line. Finally, molecular docking analysis verified that the erlotinib moiety of compound **CE17** could form hydrogen bond with Met-769 and occupy active cavity of EGFR-TK. Therefore, we believed the integration strategy between heptamethine cyanine dyes and EGFR-TKI will contribute to enhancing the therapeutic effect of EGFR-TKI for NSCLC treatment.

Non-small cell lung cancer (NSCLC) is considered as a highly heterogeneous disease that is still difficult to treat.[1] The traditional treatment of NSCLC at advanced stage is mainly combination chemotherapy or radiotherapy. However, the advent of targeted drugs provides hope for the treatment of NSCLC.[2] Epidermal growth factor receptor tyrosine kinase (EGFR-TK) is a crucial target for NSCLC with EGFR gene mutations treatment and is also one of the key factors for adult embryo generation, tissue regeneration and organ maturation. EGFR-related proteins are involved in three important tumorigenic signaling pathways, including RAS/RAF/MAPK, PI3K/AKT/mTOR and JAK/STAT signaling.[3,4] EGFR-TK inhibitors (EGFR-TKIs) have been used for the clinical treatment of EGFR-mutated NSCLC patients.[5,6] However, EGFR-TKIs need to enter the cancer cell to block the transduction of mutated EGFR signals through competitively inhibiting the binding between adenosine triphosphate (ATP) and tyrosine kinase receptors, thus inhibiting cancer cell proliferation.[7] Hence, the improvement of tumor cell targeting and the transmembrane ability of EGFR-TKIs might enhance their therapeutic effect and even reduce side effects.

Heptamethine carbocyanine dye is a kind of near infrared fluorescent (NIFR) dye, which has been widely applied in tumor imaging *in vivo*.[8–13] Recent evidence suggests that several heptamethine cyanine dyes including IR-780, IR-783 and IR-808 have preferential abilities to target cancer cells and have the ability to accumulate in the tumors.[14] Relevant mechanism research shows that the preferential uptake of heptamethine carbocyanine dyes in cancer cells is related to hypoxia-inducible factor 1α/organic anion transporting polypeptides (HIF1α/OATPs) signaling pathway.[15,16] In fact, the tumor tissue hypoxic environment increases the expression of HIF1α leading to the upregulation of OATPs expression, which may mediate the transport of heptamethine cyanine dyes into tumor cells. In addition, the negative mitochondrial membrane potential of tumor cells is one of mechanism that these dyes can accumulate in tumor cells.[17,18] Therefore, heptamethine cyanine dyes have been identified as excellent cancer-targeting small molecules. In this study, some heptamethine cyanine dyes were applied to couple with EGFR-TKI erlotinib to help achieve synergistic targeting effect. As exhibited in Fig. 1, seventeen novel erlotinib-dye

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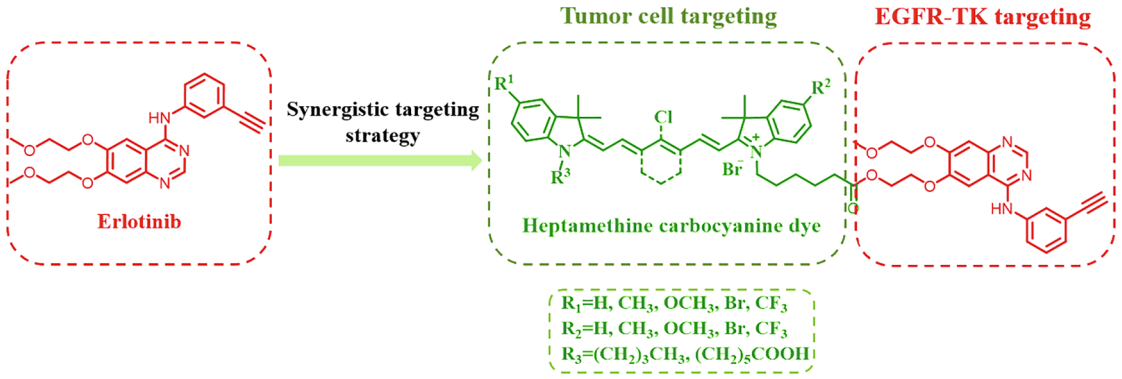
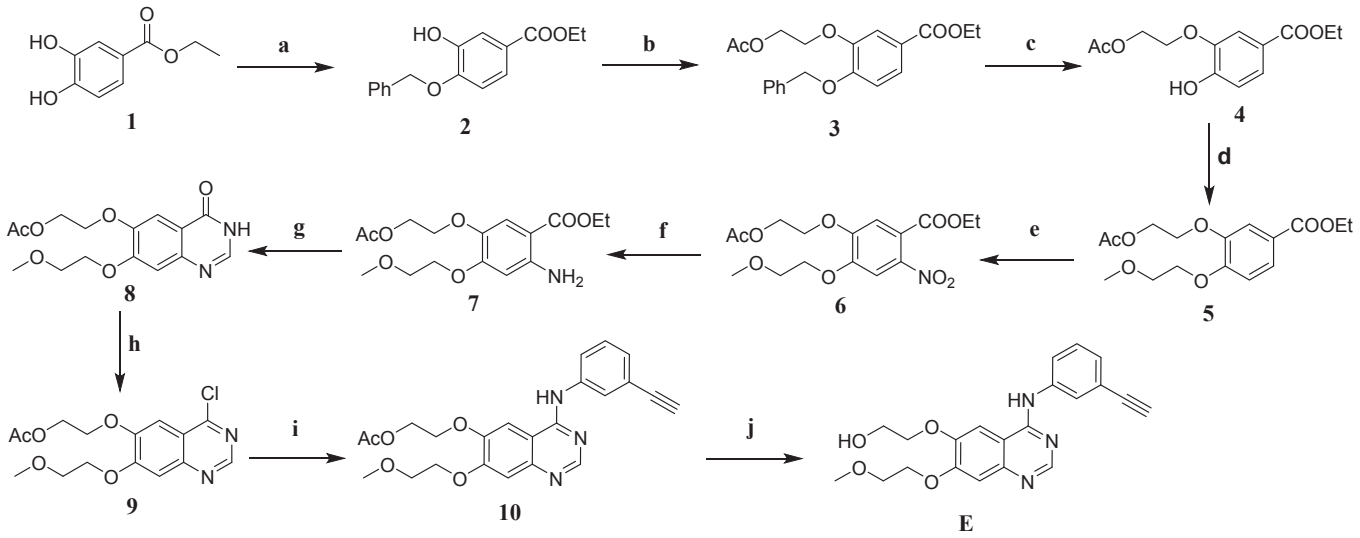
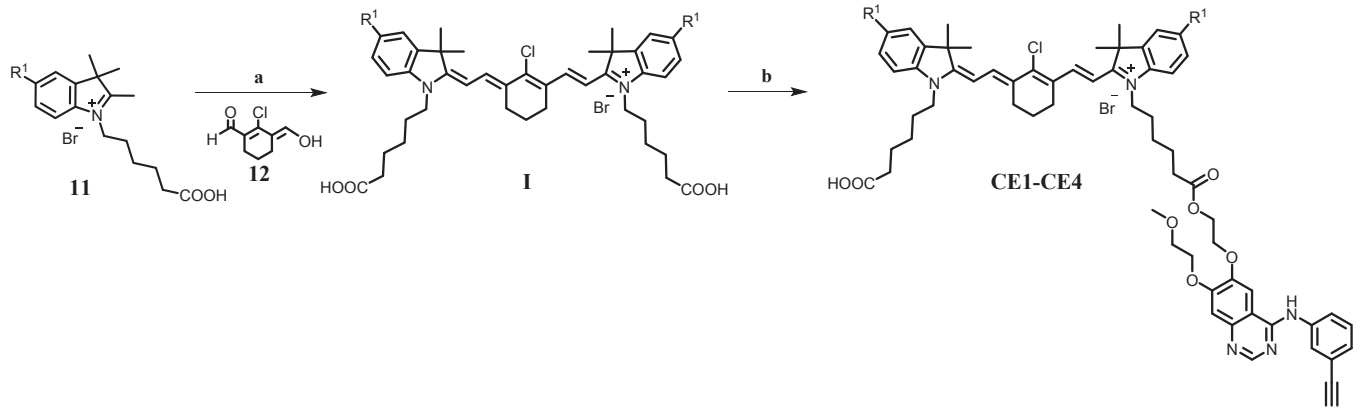


Fig. 1. Schematic diagram of the novel compounds design.



Scheme 1. Synthetic route of compound **E.** Reagents and reaction conditions: **a)** benzyl alcohol, PPh_3, DIAD, THF, −5℃ to r.t.; **b)** 2-bromoethyl acetate, K_2CO_3, DMF, r.t.; **c)** Pd/C, H_2, AcOEt, r.t.; **d)** 1-bromo-2-methoxyethane, K_2CO_3, DMF, r.t.; **e)** HNO_3, H_2SO_4, AcOH, 0℃ to r.t.; **f)** Pd/C, H_2, AcOEt, r.t.; **g)** $HCONH_2$, $HCOONH_4$, 180℃; **h)** $POCl_3$; **i)** 3-aminophenylacetylene, isopropyl alcohol, 80℃; **j)** KOH, methanol, r.t.

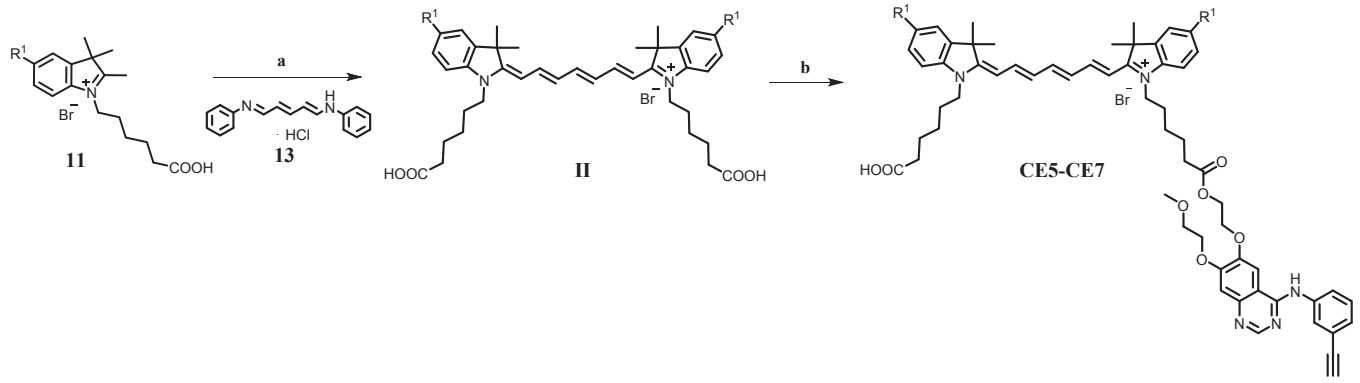


Reagents and reaction conditions: **a)** AcONa, Ac_2O, 70℃; **b)** **E**, EDCI, DMAP, CH_2Cl_2, r.t.

Scheme 2. Synthetic route of target compounds **CE1-CE4.** Reagents and reaction conditions: **a)** AcONa, Ac_2O, 70℃; **b)** **E**, EDCI, DMAP, CH_2Cl_2, r.t.

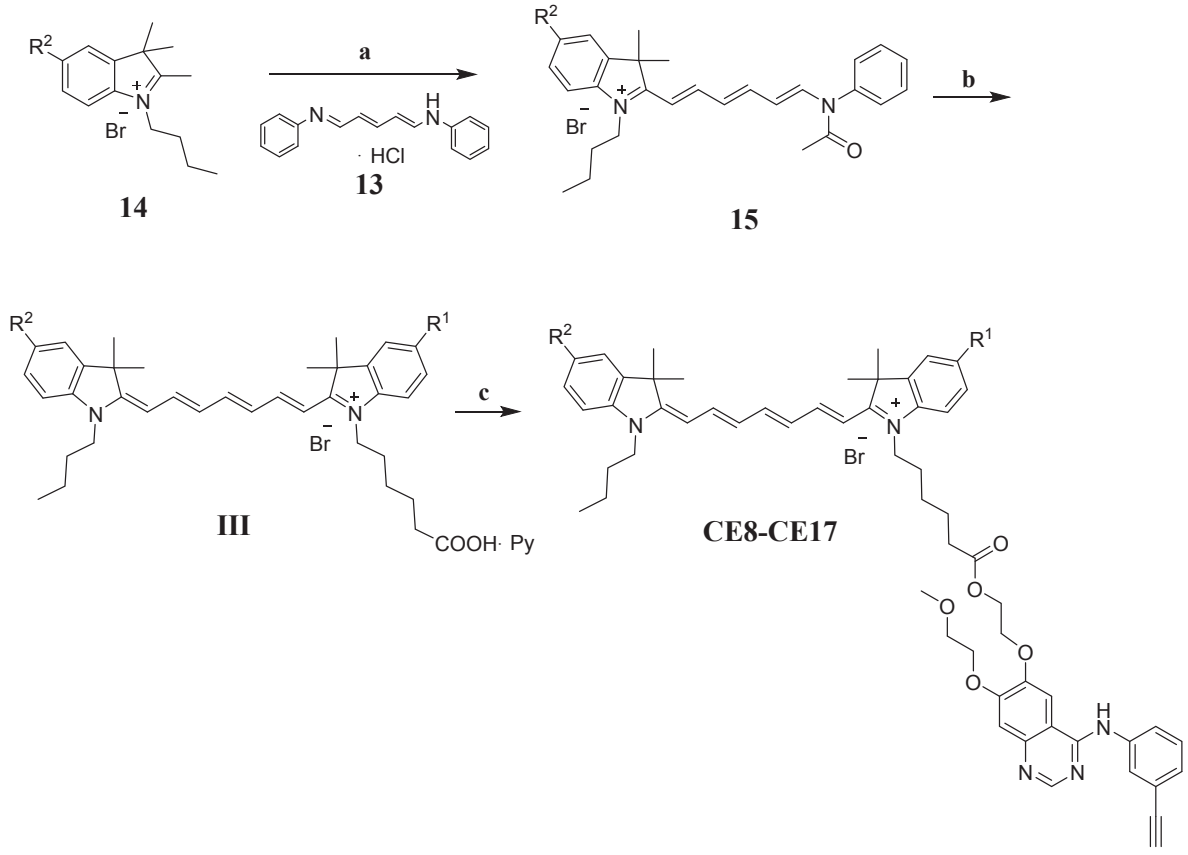
conjugates were designed and synthesized through pH-sensitive ester linkage.

Firstly, as illustrated in Schemes 1 and 2-(4-(3-ethynylaniline)-7-(2-methoxyethoxy) quinazoline-6-oxy) ethanol (**E**) was synthesized through 10-step reactions.[19] The synthesis started with commercially available ethyl 3,4-dihydroxybenzoate (**1**), which was converted into ethyl 4-(benzyloxy)-3-hydroxybenzoate (**2**) through Mitsunobu reaction. What is noteworthy is that the selective etherification reaction of catechol was achieved by a S_N2 reaction using the reagents of PPh_3 and diisopropyl azodiformate (DIAD) in the first step.[20] Etherification of the



Reagents and reaction conditions: **a)** Et_3N, Ac_2O, 70℃; **b) E**, EDCI, DMAP, CH_2Cl_2, r.t.

Scheme 3. Synthetic route of target compounds **CE5**-**CE7.** Reagents and reaction conditions: **a)** Et_3N, Ac_2O, 70°C; **b) E**, EDCI, DMAP, CH_2Cl_2, r.t.



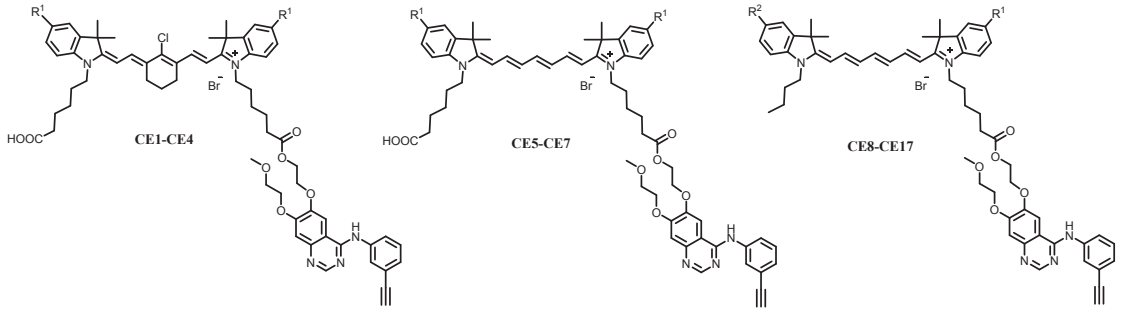
Reagents and reaction conditions: **a)** Ac_2O, 70℃; **b) 11**, pyridine, 60℃; **c) E**, EDCI, DMAP, CH_2Cl_2, r.t.

Scheme 4. Synthetic route of target compounds **CE8**-**CE17.** Reagents and reaction conditions: **a)** Ac_2O, 70°C; **b) 11**, pyridine, 60°C; **c) E**, EDCI, DMAP, CH_2Cl_2, r.t.

phenol in **2** was carried out with 2-bromoethyl acetate, producing the intermediate **3**. The benzyl group was removed under Pd/C and H_2 conditions. The product **4** was alkylated with 1-bromo-2-methoxyethane to obtain **5**. After the nitration of **5**, the nitro group in **6** was reduced by Pd/C and H_2. The quinazolin heterocycle in **8** was formed by the intermediate **7**, formamide and ammonium formate. The halogenation of phenolic hydroxyl group in **8** produced intermediate **9**. The nucleophilic substitution of **9** was conducted in the presence of 3-aminophenylacetylene. Finally, the product **10** was hydrolyzed with KOH, resulting in the formation of compound **E**.

The structural modification of heptamethine cyanine dyes includes the substitution of different groups on the C-5 or N-1 position of the indole structure and the introduction of rigid cyclohexenyl ring in the middle of the methine chain. The quaternary ammonium compound **11** was synthesized according to our previous study (see in Scheme S2).[21] Heptamethine cyanine dyes **I** (Scheme 2) were obtained by an one-step Aldol reaction between compound **11** and 2-chloro-3-(hydroxymethylene)cyclohex-1-enecarbaldehyde (**12**) with the addition of base (AcONa).[22] And the intermediate **12** was synthesized by Vilsmeier-Haack reaction using $POCl_3$, DMF and cyclohexanone as reacting

Table 1
Anti-proliferation on tumor cells (μM) of test compounds in A549, H460, H1299 and MDA-MB-231 cells.



Compd.	R^1	R^2	Cytotoxicity IC_{50} (μM)			
			A549	H460	H1299	MDA-MB-231
CE1	H	–	9.04	15.20	6.96	4.31
CE2	CH_3	–	2.38	0.816	1.55	3.36
CE3	OCH_3	–	1.96	2.70	2.21	4.23
CE4	Br	–	7.33	13.66	17.58	0.609
CE5	H	–	1.13	7.25	5.14	1.62
CE6	CH_3	–	2.10	5.65	4.15	6.21
CE7	OCH_3	–	2.59	5.12	14.94	3.30
CE8	H	H	0.631	0.57	2.00	< 0.125
CE9	CH_3	H	2.07	2.12	1.74	0.313
CE10	OCH_3	H	3.94	2.47	0.68	< 0.125
CE11	Br	H	0.579	0.655	0.693	< 0.125
CE12	CF_3	H	0.23	0.813	0.541	0.356
CE13	H	OCH_3	0.388	0.549	0.378	0.306
CE14	CH_3	OCH_3	0.678	0.827	1.348	0.531
CE15	OCH_3	OCH_3	0.455	0.217	0.11	< 0.125
CE16	Br	OCH_3	2.08	0.855	0.246	0.833
CE17	CF_3	OCH_3	0.177	0.301	0.079	0.311
Erlotinib	–	–	2.63	25.32	28.92	15.51
Taxol	–	–	< 0.001	< 0.001	0.005	< 0.025

reagents. It is noted that there is no need to purify intermediate **I** after the reaction was finished. The dyes (**I**) can be acquired just through diluting the acetic anhydride solvent with water and filtration. Compound **CE1-CE4** were prepared by the reaction of the intermediates **I** and **E** with the addition of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI) and DMAP.

Different from the synthetic route of **I**, the Aldol reaction was carried out at the presence of **11**, N-(5-anilino-2,4-pentadienylidene)aniline hydrochloride (**13**) and EtN_3 as the base when producing heptamethine cyanine dyes **II**. Similarly, the reaction between compound **II** and **E** could provide conjugates **CE5-CE7** (Scheme 3).

The general synthetic strategy for the formation of the target compounds **CE8-CE17** is shown in Scheme 4. Asymmetric heptamethine cyanine dyes **III** were obtained by two-step Aldol reaction of **14**, N-(5-anilino-2,4-pentadienylidene)aniline hydrochloride (**13**) and **11**. The synthetic route of quaternary ammonium compounds **14** is similar to **11**. However, there is no need to add base in the first step Aldol reaction, and the pyridine serves as the solvent directly in second step Aldol reaction. Finally, compounds **CE8-CE17** were synthesized by the reaction of dye **III** and **E**. The structure of the above target compounds was confirmed by NMR spectra and high resolution mass spectrum.

Subsequently, the anti-proliferation effect of compounds **CE1-CE17** on tumor cells by MTT assay (erlotinib and taxol as the control drugs). EGFR is highly expressed in lung cancer and breast cancer. Therefore, human lung adenocarcinoma cells A549, human large cell lung cancer cells H460, human lung cancer cells H1299 and human breast cancer cells MDA-MB-231 were used to investigate the antiproliferative activity of compounds **CE1-CE17**. The results are shown in Table 1.

The following SAR can be gathered from the data shown in Table 1. Firstly, all compounds exhibited superior antiproliferative activity against H460, H1299 and MDA-MB-231 cells, and most compounds (except **CE1**, **CE4** and **CE10**) displayed better antiproliferative activity over A549 cells in contrast with erlotinib. Secondly, the antiproliferative activity of compounds **CE8-CE17** is better than compounds **CE1-CE7**, indicating that heptamethine cyanine dye might possess superior tumor targeting ability when N-1 position is butyl. Thirdly, the antiproliferative activity of the conjugates could be

Table 2
The cytotoxicity of CE8 and CE17 on MCF-10A cells.

Compd.	IC_{50} (μM)
CE8	33.2
CE17	38.6

Table 3
The inhibition effect of test compounds on EGFR activity in A549 cells.

Compd.	IC_{50} (μM)
CE12	0.205
CE17	0.124
Erlotinib	5.182

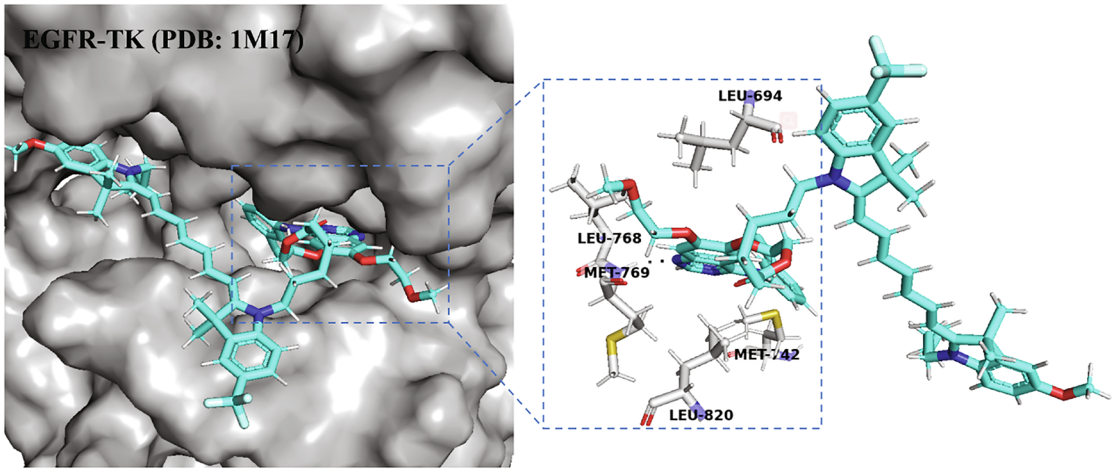


Fig. 2. Stereoview of the binding site and nearby residues from compound **CE17** in EGFR-TK (PDB: 1M17).

significantly improved when the central methine chain is lack of the rigid cyclohexene ring. Finally, surprisingly, the IC_{50} of compound **CE17** reduced approximately 14-fold (IC_{50} = 0.177 μM) compared with the parent drug erlotinib (IC_{50} = 2.628 μM) in A549 cells, indicating that the trifluoromethyl might enhance the antiproliferative activity of above conjugates.

To verify whether the combination of EGFR-TKIs with the heptamethine cyanine dye will improve their tumor cell targeting and the transmembrane ability, we investigated the cytotoxicity effect of representative compounds (**CE8** and **CE17**) on MCF-10A cells (human normal mammary epithelial cells). As shown in Table 2, the compounds **CE8** and **CE17** demonstrated relatively weak cytotoxicity on MCF-10A cells, suggesting the *in vitro* tumor cells selectivity of the representative compounds could be enhanced.

As a result, the activities of most compounds are more excellent, which demonstrates that integrating heptamethine cyanine dye with EGFR-TK inhibitor perhaps enhance the antiproliferative effect by the synergistic targeting to tumor cells.

Since the direct test of the inhibitory effect of above compounds on EGFR-TK makes little sense, we chose to investigate the cancer cells EGFR-TK inhibiting effect of above compounds *in vitro*, so as to better reflect the transmembrane effect of the target compounds. A549 cells were selected to determine the EGFR-TK inhibiting activity of compounds **CE12** and **CE17** with the best antiproliferation activity in A549 cell. Therefore, the inhibition of intracellular EGFR-TK activity was assessed by human EGFR ELISA Kit (Boster Biological Technology, China, EK0327). According to the instructions of the kit, after treating with the concentration of 0.1 μM, 5 μM, 50 μM, 200 μM, and 1000 μM of compounds **CE12**, **CE17** and erlotinib 30 h, A549 cells were lysed. The protein level was determined by the bicinchoninic acid (BCA) method, and the EGFR activity of the cells was measured by the ELISA method. The absorbance (OD value) at 450 nm was measured using a microplate reader (US Synergy-HT). The results are shown in Table 3. Compounds **CE12** and **CE17** can both inhibit EGFR-TK activity, and compound **CE17** exhibited more excellent EGFR-TK inhibition than compound **CE12** and erlotinib, which is consistent with the MTT results. Therefore, the antiproliferative activity and EGFR-TK inhibitory activity of compound **CE17** are superior to erlotinib, implicating that the antitumor effect is probably associated with the increased intracellular concentrations of target conjugates due to their enhanced targeting and transmembrane ability to tumor cells.

Finally, in order to better understand and verify the potential action mode of compounds with EGFR-TK, the molecular docking was conducted. Compound **CE17** was chosen to dock with EGFR-TK (PDB: 1 M17). As exhibited in Fig. 2, there is an H-bond interaction between the N-1 of the quinazoline moiety in **CE17** and Met-769 of 1 M17. In addition, the erlotinib moiety of compound **CE17** successfully enters into the active hydrophobic cavity (a hydrophobic pocket with the surrounding residues like Leu-694, Met-742, Leu-768 and Leu820, and the N-1 of the quinazoline accepts an H-bond from the Met-769 amide nitrogen),[23] while the heptamethine cyanine dye moiety locates at the outside of the cavity. Thus, above results might interpret the possible binding mode between compound **CE17** and EGFR in tumor cells.

In conclusion, we designed and synthesized a series of novel heptamethine carbocyanine dyes-erlotinib conjugates. Most compounds exhibited superior antiproliferative activity against A549, H460, H1299 and MDA-MB-231 cell lines compared with the parent drug erlotinib. Meanwhile, representative compounds (**CE8** and **CE17**) exhibited weak cytotoxicity on human normal mammary epithelial MCF-10A cells. Moreover, the conjugate **CE17** also showed higher EGFR-TK inhibition activity (IC_{50} = 0.124 μM) than erlotinib (IC_{50} = 5.182 μM) in A549 cell line. Together with the result of the antiproliferative activity assay and EGFR-TK inhibition assay, erlotinib conjugates probably enhanced their antitumor activity by synergistic targeting effect. Moreover, the molecular docking results were consistent with the suppression of tumor cells. Therefore, the conjugation of tumor cell targeting heptamethine dyes and EGFR-TKI will be an attractive approach for the antitumor drug design.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data (synthetic procedures and NMR spectra of compounds **CE1**-**CE17**) to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127557.

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