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14. ABSTRACT

The goal of this proposal was to develop low molecular weight inhibitors of EGFR that disable receptor functioning by preventing critical activating transitions of the extracellular domain. The lead compound, EL1-FD1, has been designed and found able to reverse the malignant properties of EGFR transformed cells in vitro and in vivo. During the reporting period, the designed EGFR ectodomain inhibitor has been tested for inhibition of EGFR phosphorylation and growth of breast cancer cells in vitro and in vivo. The compound has been shown to inhibit EGF induced phosphorylation of EGFR in EGFR overexpressing cells. A strong inhibitory effect of the compound against MDA-MB-468 cells has been demonstrated in a poly-HEMA assay. The compound effectively inhibited tumor growth in mice with MDA-MB-468 and A431 xenografts and had a moderate effect in MDA-MB-231 xenografts in vivo. Crystallization studies have produced two types of EGFR crystals suitable for soaking experiments with the inhibitory compounds. A number of structural analogs have been designed that have similar antitumor activities, but more favorable pharmacological properties, compared to the lead compound.

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EGFR inhibitor, antitumor activity

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INTRODUCTION.

EGFR represents an important target for breast cancer therapeutics. The clinically used small molecule EGFR inhibitors targeted to the kinase domain of the receptor have poor biological activity in patients with WT (wild type) EGFR. Using structure based approaches developed in our laboratory, we have designed low molecular weight compounds targeted to the ectodomain of EGFR that act by inhibiting conformational rearrangements required for receptor activation. During the reporting period, the lead compound EL1-FD1 has been tested for inhibition of EGFR phosphorylation and growth of breast cancer cells *in vitro* and *in vivo*. We also made significant advances in lead optimization and crystallography studies.

BODY.

Effect of EL1-FD1 on EGFR phosphorylation.

EL1-FD1 has been tested for its effect on receptor phosphorylation in EGFR overexpressing NE91 cells (Fig. 1). The compound effectively inhibited EGFR phosphorylation.

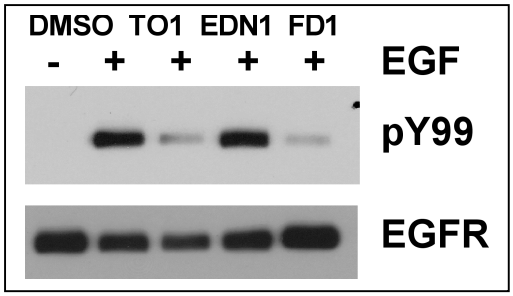
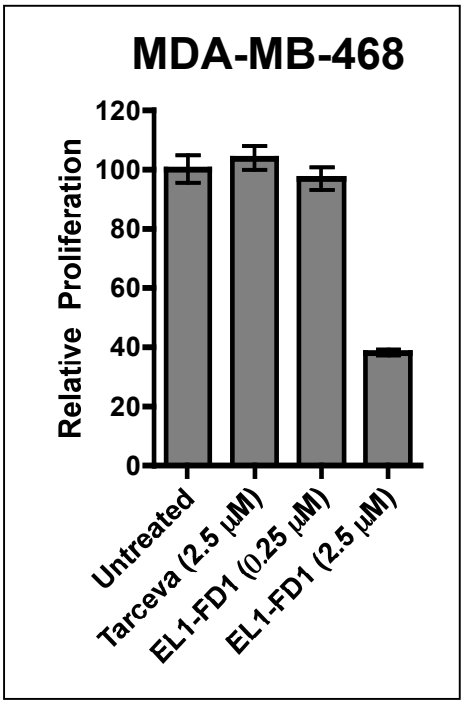


Fig. 1. Effect of EL1-FD1 on EGFR phosphorylation in NE91 cells. Western blot analysis shows effect of EL1-FD1 and some other compounds on EGFR phosphorylation in the presence of 50 ng/ml EGF. Total EGFR levels in all samples are shown as a control.

Effect of EL1-FD1 on tumor cell proliferation.

EGFR ectodomain inhibitor EL1-FD1 has been tested against MDA-MB-468 breast cancer cells in a polyHEMA based proliferation assay (Fig. 2).

Figure 2. Effects of the EGFR ectodomain inhibitor EL1-FD1 on cell proliferation in human breast cancer cell line MDA-MB-468. 5 mg/ml Poly(2-hydroxyethyl methacrylate) (PolyHEMA) powder (Sigma) in 95% ethanol was dissolved at 50°C, filtered, and 200 ul was pipeted into each well of 96-well flat-bottom plates. Plates were dried at 50°C overnight in a dry incubator. Wells were rinsed with PBS and pre-moistened with 50 ul of cell culture media. Six thousand cells were added per well. The compound was added the following day to each well and the plates were incubated for 72 hrs in a humidified 37°C incubator with 5% CO2. Alamar blue (Serotec) indicator dye (7%) was added to each well and incubated for 2-4 hrs until the dye turned from blue to purple/red. A spectrophotometer (SPECTRA Fluor, Tecan) was used to measure the colorimetric dye at wavelengths of 530 nm (ex) / 595 nm (em) and results were normalized to 100%. Bars represent mean (n=6) +/- SEM.



The breast cancer cell line MDA-MB-468 is known to be resistant to TKI inhibitors, including Tarceva (used as a control in Fig. 2), but susceptible to anti-EGFR antibodies. In contrast to the kinase domain inhibitor Tarceva, the ectodomain targeted inhibitor EL1-FD1 demonstrated inhibition of tumor cell proliferation in this cell line *in vitro* (Fig. 2).

***In vivo* studies.**

To test the antitumor activity of EL1-FD1 against breast cancer *in vivo*, we have treated MDA-MB-468 and MDA-MB-231 tumor xenografts with the inhibitor. MDA-MB-468 cells (5 x 10^6) were inoculated into nude mice in 100 μl of Matrigel/DPBS (1:1 vol) (BD Matrigel™ Basement Membrane Matrix; BD Biosciences, San Jose, CA). Five days after inoculation, mice were divided into the control and the EL1-FD1 treatment groups (4-6 mice each group). EL1-FD1 was administrated by i.p. injection at a dose of 15 mg/kg, three

times per week. Significant shrinkage of tumor volume was observed in the EL1-FD1 treatment group. At day 23, when tumor growth in the control group started to accelerate, two mice in the control group were selected as the "switch" group to receive the inhibitor treatment at the same dose. Tumors in the switch group appeared to respond to EL1-FD1 treatment initially, although they started to grow again after one week. However, the tumors in the switch group were still smaller than those in the control group.

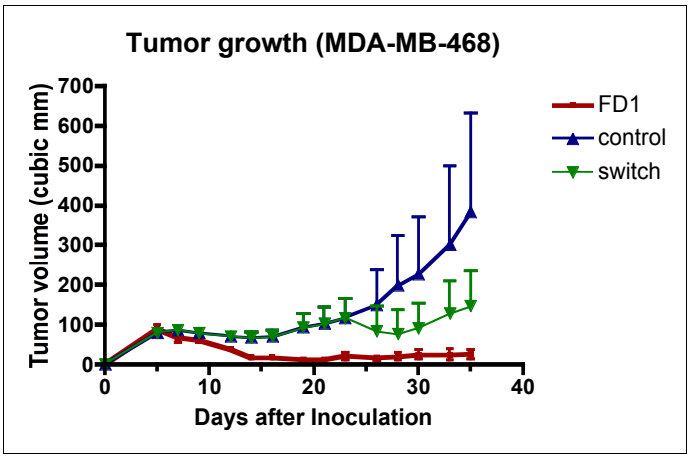


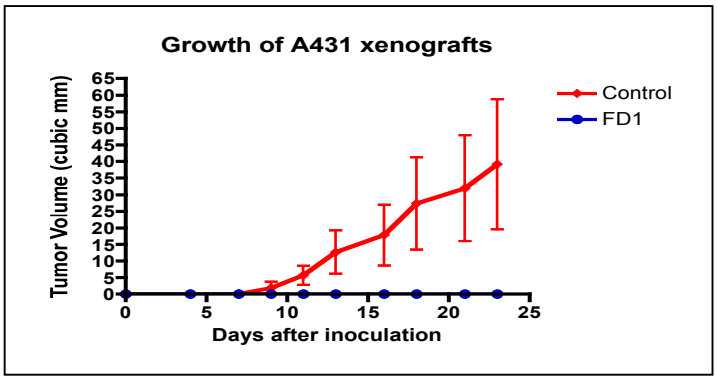
Figure 3: Tumor growth in response to FD-1 treatment. Nude mice were inoculated with 5 x 10^6 MDA-MB-468 cells into the flank. Mice bearing established tumors were treated with FD1 (15 mg/kg, i.p., 3 times/week).

The experiment has demonstrated that the lead compound EL1-FD1 has a strong antitumor activity against breast cancer when used *in vivo*.

We next performed MDA-MB-231 xenografts with 1 x 10^7 cells inoculated into nude mice in 100 μl DPBS. We began treatment at day 9. In this pilot experiment with 2-3 mice per group, we also observed some activity of EL1-FD1 in the inhibition of tumor growth (not shown). Additional experiments with more mice in each group subsequently confirmed the activity.

In the past year we have identified ways to improve the solubility of ELI-FD1 for *in vivo* application. We examined the activity of EL1-FD1 in an aggressive human tumor driven solely by EGFR over-expression. This small molecule has extraordinary activity *in vivo*. The EL1-FD1 small molecule has a Mr of ~400 and completely arrests growth of human A431 tumor cells *in vivo* but has no activity against tumor cells which are not transformed by EGFR.

Figure 4. Growth of A431 tumor cells is dramatically inhibited by EL1-FD1. Athymic nude mice were injected s.c. with 1x10^6 A431 cells on the flank. EGFR inhibitor EL1-FD1 was administered by i.p. injection at a dose of 15 mg/kg, three times per week beginning on day 4. The data are presented as mean tumor volume (mm3) ±SEM.



ITC studies.

ITC studies of the receptor – inhibitor binding affinity have been attempted but could not be completed because of the low solubility of the lead compound. Reverse titration of the inhibitor by the protein generated the first few titration peaks confirming the binding of the inhibitor to the receptor, but the experiment could not be finished due to insufficient concentration of the receptor. Structural analogs of the lead compound with improved solubility properties have been designed and will be used for the ITC studies in the future.

Structural analogs of EL1-FD1.

A number of structural analogs of EL1-FD1 have been designed and synthesized for optimization of receptor binding properties, biological activity and pharmacological properties of the lead compounds. The

designed EL1-FD1 analogs were tested for inhibition of EGFR phosphorylation in breast cancer cell lines. Two compounds, EL1-FD1A and EL1-FD1B, had similar antitumor properties and improved solubility properties compared to the lead compound EL1-FD1. These improved analogs provide a foundation for the future biophysical and crystallography experiments.

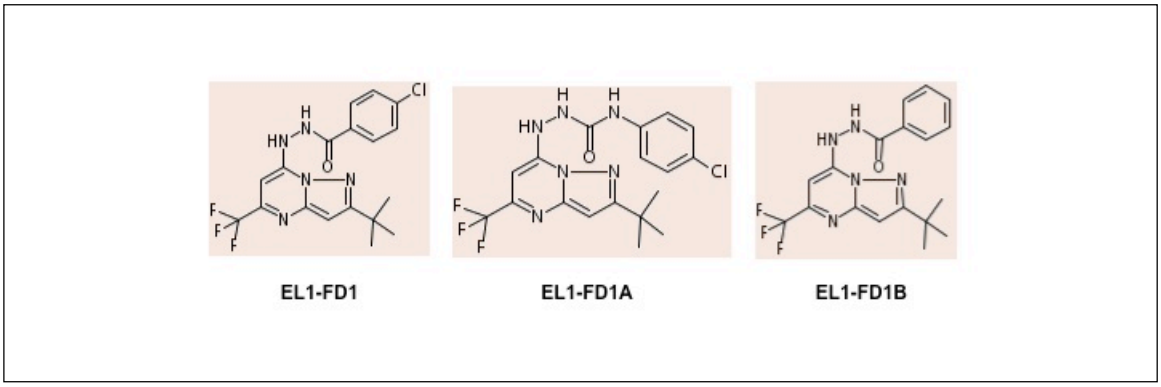


Figure 5. EL1-FD1 and analogs. The molecular weights are: EL1-FD1: 411.81, EL1-FD1A: 426.83, and EL1-FD1B: 377.37.

Crystallization studies.

A major question that must be addressed in assessing the designed inhibitors is whether they bind to the receptor in the intended manner. The most direct way to do this is to determine the crystal structure of the complex of sEGFR in complex with the small molecule. We tried to address this question by co-crystallizing sEGFR with the inhibitor EL1-FD1. However, no crystals could be obtained using this method. We therefore decided to use the compound soaking approach. Our first step was to prepare sEGFR crystals that could be used in the soaking experiments.

We can grow crystals of sEGFR in the autoinhibited or "tethered" monomeric form under two different conditions, at low pH in the presence of EGF and at neutral pH in the absence of ligand.

Crystals of sEGFR:EGF complex at pH 5.0. Crystals of sEGFR:EGF (Fig. 6) are grown at pH 5.0. At this pH, EGF binds to sEGFR with very low affinity and does not promote receptor dimerization. Since sEGFR is known to be in the tethered conformation under these conditions, the obtained crystals would be suitable for soaking experiments with the inhibitors that are predicted to bind to the tethered form of the receptor such as EL1-FD1 and its analogs.

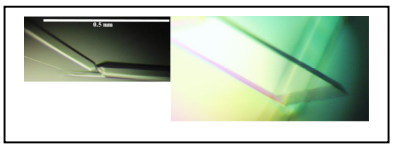


Figure 6. Examples of crystals of sEGFR:EGF grown at pH 5.0. The bar represents a distance of 0.5 mm. Average crystal dimensions: 1 x 0.1 x 0.08 mm.

Crystals of tethered sEGFR in the absence of ligand. To date we have been unable to grow crystals of isolated sEGFR. However we have been able to grow crystals of sEGFR in complex with the Fab fragments from several inhibitory antibodies. For our soaking experiments we prepared the crystals of sEGFR in complex with the Fab fragment of cetuximab/Erbitux where sEGFR is in a tethered conformation. These crystals will also be used for small molecule soaking experiments.

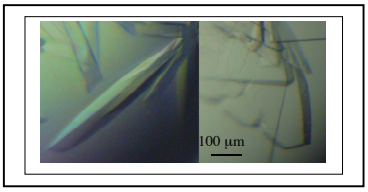


Figure 7. Crystals of Fab:sEGFR grown at neutral pH. The bar represents a distance of 100 mm.

KEY RESEARCH ACCOMPLISHMENTS.

- The biological activity of the lead compound against breast cancer tumors has been demonstrated *in vitro* and *in vivo*.
- We have extended the biological studies on EL1-FD1 to A431 tumors driven solely by EGFR and have observed dramatic and complete inhibition of tumor growth *in vivo*.
- A number of the modified lead compound analogs with similar biological activity and improved solubility properties have been designed and synthesized.
- Two different types of crystals that will be used in crystallography studies of the receptor-inhibitor complex have been produced providing a foundation for the future crystallography studies.

REPORTABLE OUTCOMES.

A manuscript describing the obtained results is in preparation.
The grant helped Dr. Berezov obtain a position at Cedars Sinai Medical Center.

CONCLUSION.

During the reported period, we have demonstrated the biological activity of the lead compound in breast cancer tumors *in vitro* and *in vivo*, which was one of the main goals of the project. Modified analogs of the lead compound with similar biological activity and improved solubility properties have been generated. This part of the work provided a foundation for further biological activity studies with the lead and the modified compounds. Significant advances have also been made for the successful accomplishment of the crystallography studies.

We believe that with improvement of Kd and increase in solubility the compound set may have therapeutic applications in the future.

REFERENCES AND PERSONNEL.

Dr. Alan Berezov presented a poster at the 2011 Era of Hope meeting entitled, “Small Molecule Inhibitors of EGFR Ectodomain for Breast Cancer Therapy.”