Translational Oncology 14 (2021) 101140

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

Disruptin, a cell-penetrating peptide degrader of EGFR Cell-Penetrating Peptide in Cancer Therapy

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ARTICLE INFO

Keywords:
HNSCC
Lung Cancer
EGFR
Cell-Penetrating Peptide

ABSTRACT

Disruptin is a cell-permeable decoy peptide designed to destabilize activated EGFR, both by inhibiting Hsp90 chaperoning and dissociating the active asymmetric EGFR dimer, which leads to an increase in engagement of activated EGFR with the proteolytic degradation machinery and subsequent loss from the cells. Disruptin is an N-terminally biotinylated nonadecapeptide, with 8 amino acids from the αC-helix-β4 sheet loop of EGFR (S^{767}-C^{774}) fused to a TAT undecapeptide. The S^{767}-R^{775} loop is at the interface with juxtamembrane domains in the active EGFR dimers and is a binding site for Hsp90. Cellular studies in EGFR-activated tumor cells demonstrated that Disruptin causes the disappearance of EGFR protein from cells over a few hours, a growth inhibitory effect, similar but more effective than the EGFR kinase inhibition. Interestingly, cells without activated EGFR remained unaffected. *In vivo* studies showed that Disruptin slowed the growth of small tumors. Larger tumors responded to intratumoral injections but did not respond to systemic administration at tolerated doses. Investigation of these results revealed that systemic administration of Disruptin has acute toxicities, mainly related to its TAT peptide moiety. Therefore, we conclude that although the efficacy of both *in vitro* and *in vivo* intratumoral injection of Disruptin supports the therapeutic strategy of blocking activated EGFR dimerization, Disruptin is not suitable for further development. These studies also highlight the importance of the chosen models and drug-delivery methods for such investigations.

Introduction

The Epidermal Growth Factor Receptor (EGFR) is a receptor tyrosine kinase (RTK) implicated in oncogenesis in a wide variety of tumors. It is the most studied of a family of four RTKs, the erbB-1–4 family[1]. Like other RTKs, the erbB family exists in the plasma membrane as monomers, dimerizing (homo/hetero) when their extracellular domains bind the appropriate ligands[2, 3], causing the kinase to switch from an inactive to an active conformation. When EGFR binds its cognate ligands, conformational changes, passed from the extracellular ligand-binding domain through the transmembrane and juxtamembrane domains, lead to asymmetric dimerization of their intracellular kinase domains, causing one of the two kinase domains to switch from an inactive to an active conformation[4]. This conformation allows binding with both ATP and substrate, and the kinase then phosphorylates tyrosine hydroxyls[5–7].

Artificial mutation in the ATP binding domain (K^{745} also referred to as K^{721}) inhibits EGFR phosphorylation and downstream signaling[8]. These observations led to the idea of blocking EGFR kinase activity using small molecules[9]. Mouse genetic studies have shown that complete loss of EGFR is embryonic lethal, suggesting EGFR has a housekeeping function[10]. The waved-2 mouse, exhibiting phenotypes like unusual skin, fur, and eye abnormalities, comprises a point mutation, V765G, in the EGFR tyrosine kinase domain within the αC-helix. V765G EGFR is a severe hypomorph causing up to a 90% reduction in EGFR phosphorylation, suggesting an important function of the αC-helix[11]. Subsequent studies indicated that the αC-helix is critical for the structural integrity of the activated kinase conformation[12]. However, the waved-2 mice exhibit a relatively mild phenotype despite the 90% reduction in EGFR kinase activity. Based on these findings, we hypothesized that the ablation of the EGFR protein would have more significant effects than a kinase inhibitor in controlling EGFR-driven tumors.

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https://doi.org/10.1016/j.tranon.2021.101140
Received 11 March 2021; Received in revised form 20 May 2021; Accepted 27 May 2021

Geldanamycin (GDN) was initially identified as an inhibitor of RTKs, especially erbB2. However, subsequent studies showed that GDN does not bind to kinases; rather, it is a potent inhibitor of the chaperone protein Hsp90. When Hsp90 is inhibited, erbB-2 is rapidly degraded in the cells[13]. The loop between the αC-helix and the β4-sheet in the N-lobe of erbB-2 (M^{774}-R^{784}) (aka M5 loop) is the primary binding site for Hsp90[14]. This loop is located on the rear face of the kinase and acts as a back wall to the catalytic cleft. EGFR has a slightly shorter but largely superimposable M5 loop (S^{768}VDNPHVCR^{776}) (see PDB#s 3POZ and 3RCD). It has a lower affinity for Hsp90 than the M5 loop of erbB-2, but recent studies have shown that Hsp90 inhibition reduces the half-life of EGFR in cells by more than 50%[13]. We generated an EGFR construct in which the first 6 amino acids of the M5 loop were scrambled to N^{768}HVPSD^{773} (Scram-EGFR)[15]. We found that Scram-EGFR expression in EGFR-null CHO cells was much lower compared to wt-EGFR, although mRNA levels were similar. Furthermore, Scram-EGFR formed very few dimers, even upon EGF stimulation of cells [16].

Based on these observations, we generated two peptides, S^{768}VDNPHVC^{775} (Disruptin) and N^{768}HVPSDVC^{775} (Scram), both of which were N-terminally biotinylated to aid with bioanalytical work and C-terminally attached to the cell permeating peptide (CPP) TAT sequence, YGRKKRRQRRR, to form Biotin-YGRKKRRQRRRSVDNPHVC (Disruptin) and Biotin-YGRKKRRQRRRNHVPSDVC (Scram, control). In a previous study, we found that Disruptin was effective against UMSCC1 xenografts if treatment was initiated when tumors were about ~30 mm^3[15, 16].

In this study, we found that systemic injection of Disruptin is effective against EGFR-TKI resistant smaller tumors, but this treatment was minimally effective in animals with established tumors. The CPP conjugated peptide showed dose-limiting toxicities when dosed systemically. We further showed that intratumoral injections of Disruptin reduced EGFR protein and micro-blood vessel densities and slowed tumor growth. Overall, these findings suggest that an agent that reduces EGFR protein level could offer an alternate therapy for EGFR-driven TKI-resistant tumors.

Materials and Methods

Materials

Disruptin, Biotin-NH-YGRKKRRQRRRSVDNPHVC-CO_2H, NB-Disruptin (non-biotinylated), NH_2-YGRKKRRQRRRSVDNPHVC-CO_2H, RI-Disruptin (retroinverso), NH_2-cvhpndvsrrrqrrkkrgy-CO_2H, and Scram-peptide, Biotin-NH-YGRKKRRQRRRNHVPSDVC-CO_2H were synthesized by SynPeptide Co Ltd. EGFR antibody (cat#4267) was acquired from Cell Signaling Technology, Inc. (Danvers, MA). Ki-67 antibody (ab15580) was acquired from Abcam (Cambridge, MA). CD-31 (clone JC70A) was purchased from (Dako, Carpinteria, CA). The human head and neck squamous cell carcinoma (HNSCC) cell lines, UMSCC11B, and UMSCC47 were kindly provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). The lung cancer cell line, NCI-H1975, was provided by J. Engelman (Massachusetts General Hospital, Boston, MA). A549 cells were purchased from the American Type Culture Collection (Manassas, VA). All cell lines were grown in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (Gibco, Waltham, Massachusetts) and 1X Penicillin-Streptomycin-Glutamine (Gibco# 10378016). Other reagents used in this study were Propidium Iodide (Invitrogen # P1304MP), Matrigel (BD Biosciences # 356237), Harris Hematoxylin (Leica # 3801560), Bluing Reagent (Thermo Scientific #7301), Clarifier 1 (Thermo Scientific #7401). ABC-HRP Kit (PK6100), and DAB Substrate Kit (SK-4100) were purchased from Vector Laboratories.

Clonogenic survival assay

Clonogenic survival assays were performed using a standard technique described previously[17]. Briefly, 500–2000 cells were plated onto 60 mm dishes in triplicate, and the next day, cells were treated with Disruptin or NB-Disruptin or RI-Disruptin, dissolved in distilled water. 8–12 days later, cells were fixed with acetic acid/methanol (1:7 vol/-vol), stained with crystal violet (0.5% wt/vol), and counted using a stereomicroscope. The fraction surviving each treatment was normalized to the survival of the control cells. Cell survival curves were fitted using the equation: SF = (C50) m/[(C50) m + Cm], where SF is the surviving fraction, C is the peptide concentration, C50 is the concentration of peptide that produces a 50% cell survival, and m is the slope of the sigmoid curve.

Animal studies

All animal experiments were performed according to the University of Michigan-approved protocols and conformed to their relevant regulatory standards. To generate tumor xenografts, 1×10^6 NCI-H1975 cells were transplanted into the flanks of athymic nude *Foxn1*^{nu} mice (Harlan Laboratories, Indianapolis, IN). When the tumors reached a volume of ~100 mm^3 (in approximately 5–10 days), NCI-H1975 tumor tissues were cut into small fragments in sterile conditions on ice-cold PBS. One tumor fragment was placed into the end of a pre-chilled 11-gauge trocar needle along with 50–100 μL of Matrigel® and subcutaneously implanted into the flanks of 6–8-weeks old athymic nude *Foxn1*^{nu} mice. In the intratumoral dosing experiment, only one live passage of the tumors was carried out, and smaller tumor fragments were trocar-implanted and grown to the appropriate size before treatment was initiated. In the intraperitoneal (IP) dosing experiment, a second serial passage was conducted to get more tumor material, and the implantation was repeated with large tumor fragments (~50 mg) into 6–8-weeks old athymic nude *Foxn1*^{nu} mice. The mice were randomized into two groups: control and experimental (NB-Disruptin), and when tumor volume reached ~150 mm^3 the treatment was initiated. NB-Disruptin was injected intraperitoneally at 10 mg/kg, qw . In intratumoral studies, Disruptin was injected at 10, 20 or 30 mg/kg directly into the tumor in 40 μL of saline. Tumor size was measured three times per week, and tumor volumes were calculated as follows: volume (mm^3) = $(L \times W^2)/2$. For immunohistochemical studies, mice were euthanized, tumors were harvested at various time points, and the effects on EGFR, Ki-67, and CD-31 were analyzed.

IHC staining

The Tissue and Histology Core of the Comprehensive Cancer Center and the Pathology Core for Animal Research in the Unit for Laboratory Animal Medicine at the University of Michigan assisted in preparing specimens for immunohistochemistry. After the slides were deparaffinized in xylene and rehydrated using serial ethanol dilutions, antigen site unmasking was performed by immersing the slides in citrate buffer (pH 6.0) for 20 min at high pressure and temperature inside a microwave oven. Slides were then washed in PBS, blocked for 1 h, and incubated in the primary antibody at 4°C overnight. Slides were then again washed in PBS, incubated in secondary antibody for 1 h, rewashed, and probed using DAB detection kits with an extra washing step. Slides were counterstained with hematoxylin for 1 min, post-counterstained with bluing reagent for 1 min, washed with water, and then dehydrated in ethanol and xylene before coverslip application. Images were acquired using a DS-Fi1 (Nikon, Melville, NY) camera fitted on an Olympus IX-71 microscope.

In vitro sprouting assay

Capillary sprouting was estimated as previously described in[18]. Briefly, 5×10^4 HDMEC cells/well were seeded in 24-well plates coated with growth factor reduced Matrigel. After 24 h, the cells were treated with either Scram-peptide or Disruptin (100 micrograms/ml). The

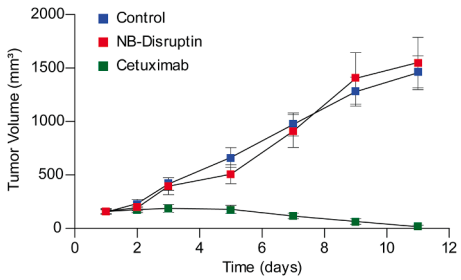

Fig. 1. Effect of Cetuximab and NB-Disruptin on NCI-H1975 xenografts. Mice bearing 150 mm^3 or larger tumors prepared from 50 mm^3 tumor fragments were treated with Cetuximab (1 mg, once weekly) or NB-Disruptin (10 mg/kg, qd), or vehicle via IP injection, and the tumor volumes were measured three times per week.

branching of capillaries was imaged on days 2, 3, and 4, and dead cells were visualized with propidium iodide staining. The live capillaries were scored (n = 5).

Statistical analysis

Significant differences between the groups were determined by one-way or two-way analysis of variance. All the statistical calculations were performed using GraphPad prism version 8.0. Significance was indicated as *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

Results and Discussion

For this study, a fresh batch of Disruptin was prepared as previously described[15]. We also synthesized Disruptin peptide minus the N-terminal biotinylated label (NB-Disruptin) and retroinverso unbiotinylated Disruptin (RI-Disruptin). The retroinverso Disruptin contains the inverted N to C sequence with all D-amino acids, *cvhpndvsrrrqrrkkrgy*. Prior to the *in vivo* experiments, the relative potencies of Disruptin, NB-Disruptin, and RI-Disruptin were confirmed (n = 2) in four cancer cell lines using a clonogenic assay, and the resulting surviving fraction at 100 μg/ml is tabulated below.

As the three forms of Disruptin were equipotent in NCI-H1975 and A549 lung cancer cells, we decided to test the efficacy of unbiotinylated peptide (NB-Disruptin) against established tumor grafts developed from NCI-H1975 tumors via trocar implantation of ~50 mm^3 fragments of *in vivo* passaged tumors. The treatment started at 150 mm^3 average tumor sizes, when it was assumed that the tumor vasculature would be well integrated into the host blood supply. Mice (11 per group) were treated with either 10, 20, and 30 mg/kg qw and 15 mg/kg bid qw for 3 weeks. Cetuximab (1 mg per week x 2) was used as an active control. The highest dose proved to be acutely toxic, killing 6/11 mice within an hour of dosing, with Benadryl offering no protection, and 1/11 mice died at the 20 mg/kg dose. All other doses were well tolerated, and no weight loss was recorded in the surviving animals. Cetuximab treatment reduced tumor volume to below palpable by Day 12. However, none of the tolerated doses of peptide showed activity statistically different from control (Fig. 1). Fig. 1 shows control and the 10 mg/kg NB-Disruptin groups, but none of the other doses examined were significantly different.

As these findings were in contrast with previous results where biotin-conjugated peptide was used, a series of experiments was carried out to understand possible reasons behind differences in *in vivo* efficacies. One possibility was that the biotinylation had played a role *in vivo*, so subsequent experiments were again conducted with Disruptin (biotinylated). Further, to avoid any systemic effects, the treatment was delivered directly into tumors grown from the injection of 10^6 NCI-H1975 cells. Animals were treated with Disruptin (5 mice per group, 10 mg/kg, IP, biw) either when the average tumor size was about 50 mm^3 or about 150 mm^3, with the results shown in Fig. 2. The results were consistent with the prior experiments in the smaller-sized tumors using systemic delivery. As seen in Fig. 2A, the small tumors showed modest effects until Day 11. After that, the treated tumors regressed and stayed around the same size until the end of the experiment on Day 14. These results were similar to those previously observed with UMSCC1 xenografts[16]. In contrast, the larger tumors showed no response to Disruptin treatment; if anything, the growth rate of treated tumors appeared faster than the controls (Fig. 2B), however, we did not investigate this further, as it seemed to be anomalous, and was not seen in any other *in vivo* experiment.

We then examined the histologic response to Disruptin treatment at a range of doses. For this experiment, female nu/nu nude mice were implanted with 1 million NCI-H1975 cells in each flank, and the tumors were allowed to grow for about 2 weeks. When tumors were in the 50–150 mm^3 range, mice were divided into 4 groups and all evaluable tumors were then injected intratumorally with either Scram peptide or Disruptin (10, 20, or 30 mg/kg in 40 μL saline). The low dose group (10 mg/kg Disruptin) had 5 mice, each with a single evaluable tumor, and both the higher dose groups (20 and 30 mg/kg Disruptin) had 4 mice, 3 of which had 2 evaluable tumors. The same dosing was repeated after 5 days, and on Day 8, the mice were evaluated for tumor growth, euthanized, and their tumors examined histologically (Fig. 3) (Table 1).

All mice survived the treatment, despite receiving up to 60 mg/kg of Disruptin in a single session (see below). The tumor doubling time was about 4 days as reported before[16] and, on average, was five-fold larger (range 3.5–8.2) on Day 8. The 10 mg/kg dose displayed no effect, but the 20 mg/kg dose exhibited slower tumor growth, with only a 2.5-fold (1.3–4.8, $p < 0.0001$) increase in size on Day 8. The 30 mg/kg data was slightly weaker, with a 3.4-fold (2.6–4.2, $p < 0.005$) increase in

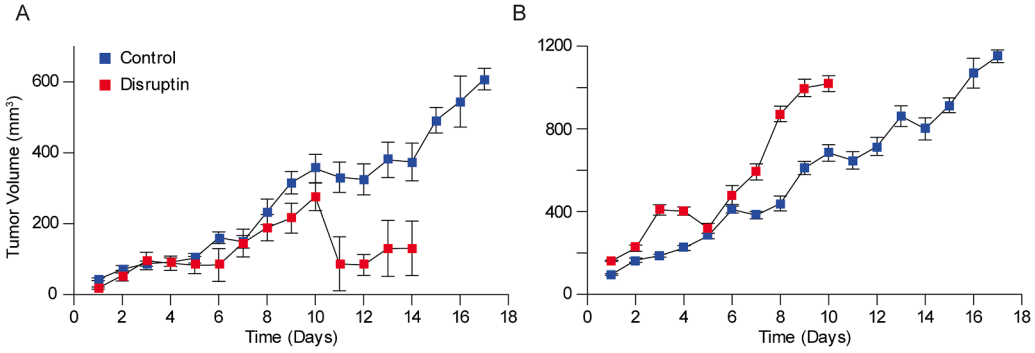

Fig. 2. Effect of Disruptin on NCI-H1975 xenografts. 1 × 10^6 NCI-H1975 cells were transplanted into the flanks of athymic nude *Foxn1*^{nu} mice (Harlan Laboratories). When the tumors reached a volume of approximately 50–150 mm^3, the mice were divided into two groups based on the average starting tumor volume of ~50 mm^3 (A) or 150 mm^3 (B). These mice were then randomized into two groups, and treatment was initiated with either Disruptin or vehicle control. A bi-weekly dose of 10 mg/kg Disruptin was given to mice in 40 μL saline directly into the tumors. Tumor volumes were recorded daily and plotted.

Data are shown as mean +/- SEM.

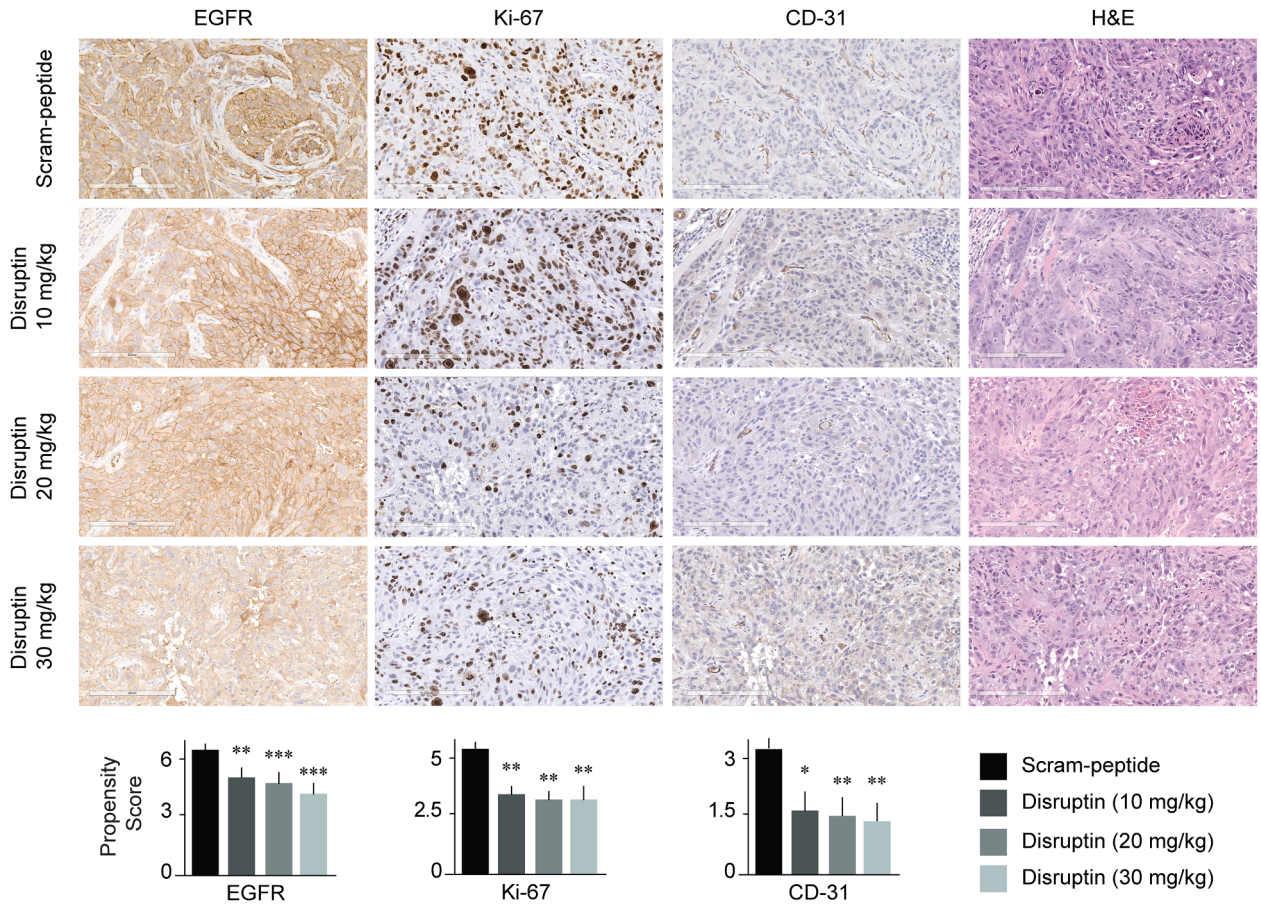

Fig. 3. Effect of Disruptin on EGFR, Ki-67, and CD-31. About 10^6 NCI-H1975 cells were transplanted into the flanks of athymic nude Foxn1^{nu} mice. When the tumors reached a volume of approximately 50–150 mm^3, the mice were randomized into four groups (1 vehicle control and 3 experimental groups), and treatment was initiated. Disruptin (10, 20, or 30 mg/kg in 40 μL saline) was injected directly into tumors on Day 1 and again on Day 5. On Day 8, the animals were euthanized, and tumors were harvested and stained for EGFR, Ki-67, and CD-31. Staining positivity was scored on a 0–5 scale and the resulting values were plotted as mean +/- SEM on a bar graph one-way ANOVA with a significance level of *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 1
Surviving fraction after treatment with Disruptin species

	NCI-H1975 (mt-EGFR)	A549 (mt-KRAS)	UMSCC11B (wt-EGFR)	UMSCC47 (wt-EGFR)
Disruptin	0.74 ± 0.09	0.80 ± 0.06	0.24 ± 0.04	0.29 ± 0.02
NB-Disruptin	0.78 ± 0.01	0.73 ± 0.06	0.40 ± 0.06	0.44 ± 0.04
RI-Disruptin	0.71 ± 0.01	0.76 ± 0.02	0.61 ± 0.06	0.69 ± 0.05

Table 2
Intratumoral Injection of Disruptin into NCI-H1975 Xenografts

Group (N)	Size D1 mg	Size D8 mg	D8/D1 ratio
Control (9)	66 ± 8.7	341 ± 45	5.34 ± 0.57
10 mg/kg (5)	78 ± 16.9	463 ± 89	6.43 ± 1.26 (ns)
20 mg/kg (7)	141 ± 28.9	385 ± 113	2.5 ± 0.46 ($p < 0.0001$)
30 mg/kg (7)	88 ± 19.7	294 ± 66	3.40 ± 0.21 ($p = 0.005$)

tumor size on Day 8. Overall, this suggests weak antitumoral activity, but certainly not enough to be useful as a single agent (Table 2).

Based on the *in vivo* outcome suggesting that the efficacy of Disruptin is limited to smaller tumors and is generally weak even when delivered directly to the tumors, we hypothesized that Disruptin affects the neovascularization required for tumors to establish. On the other hand, the established tumors or the tumors developed from transplantation of tumor fragments were resistant since they contain relatively mature blood vessels that are not significantly affected by Disruptin, resulting in minimal effects on tumor growth.

To confirm this hypothesis, the effect of Disruptin on capillary sprouting of human dermal microvascular endothelial cells (HDMEC) was assessed. HDMEC cells were seeded in growth factor reduced Matrigel, and 24 h after seeding, the cells were either treated with Disruptin or Scram-Peptide (100 μg/mL), capillary sprouting was quantitated on Days 2, 3, and 4 (Fig. 4). On Day 4, cells were stained with propidium iodide to determine cell death. Disruptin significantly reduced capillary sprouting with time, and by Day 4, no intact capillaries were observed. Moreover, at least 70% of the HDMEC cells absorbed propidium iodide indicating cell death. Scram-Peptide did not affect either capillary sprouting or cell death. These results suggest that Disruptin might affect endothelial cell viability resulting in decreased micro blood vessel densities in the tumors where blood vessels were not fully established. As shown in Fig. 3, Disruptin injection significantly reduced CD-31 staining confirming the *in vitro* observation.

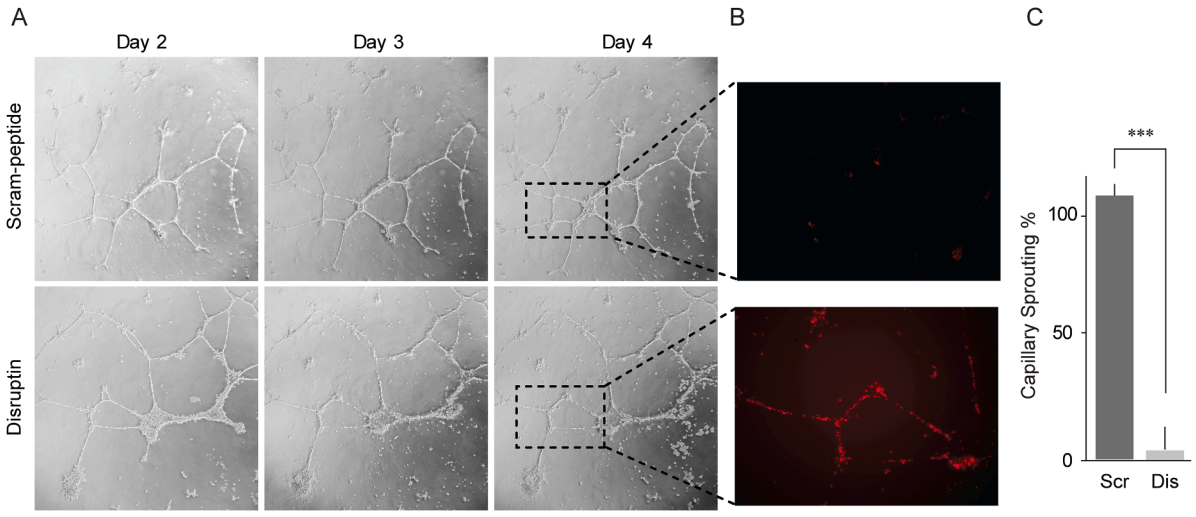

Fig. 4. Effects of Disruptin on capillary sprouting. A, Effect of Disruptin on capillary sprouting of human dermal microvascular endothelial (HDMEC) cells. B, Propidium iodide staining. C, Quantitation of live capillaries scored from 5 different wells. Differences between scram-peptide and Disruptin were assessed using two-way ANOVA with a significance level of *** $p < 0.001$.

Conclusion

Induction of EGFR degradation remains an effective approach against EGFR-TKI resistant tumors[19]. We developed Disruptin peptide that binds with EGFR, blocks EGFR dimerization, and promotes EGFR degradation[15]. Since EGFR dimerization primarily occurs upon EGFR activation, only activated EGFR is degraded upon treatment with Disruptin causing the death of EGFR-driven cells. *In vivo* data indicate that Disruptin is effective only against smaller tumors suggesting that further optimization is needed to ensure efficacy and safety of CPP conjugated peptides. Overall, these findings indicate the αC-β4 loop of EGFR is a promising target for the development of selective EGFR degraders.

Ethics Statement

Here we confirm that all the animal studies conducted were approved by the University Committee on Use and Care of Animals (UCUCA) of the University of Michigan (Protocol # PRO00006797).

Funding Source

This work was supported by the National Institutes of Health [R01 CA131290 to M.K.N.] and the Rogel Cancer Center support grant, P30CA46592. This project is a component of the Protein Folding Disease Research Initiative of the University of Michigan Medical School.

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

We thank Yee Sun Tan and the University of Michigan histology, and animal care facilities for help with several experiments. We would also like to thank Mary A. Davis for editing this manuscript.

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