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Lamellarin 14, a derivative of marine alkaloids, inhibits the T790M/C797S mutant epidermal growth factor receptor

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Abstract

The emergence of acquired resistance is a major concern associated with molecularly targeted kinase inhibitors. The C797S mutation in the epidermal growth factor receptor (EGFR) confers resistance to osimertinib, a third-generation EGFR-tyrosine kinase inhibitor (EGFR-TKI). We report that the derivatization of the marine alkaloid topoisomerase inhibitor lamellarin N provides a structurally new class of EGFR-TKIs. One of these, lamellarin 14, is effective against the C797S mutant EGFR. Bioinformatic analyses revealed that the derivatization transformed the topoisomerase inhibitor-like biological activity of lamellarin N into kinase inhibitor-like activity. Ba/F3 and PC-9 cells expressing the EGFR in-frame deletion within exon 19 (del ex19)/T790M/C797S triple-mutant were sensitive to lamellarin 14 in a dose range similar to the effective dose for cells expressing EGFR del ex19 or del ex19/T790M. Lamellarin 14 decreased the autophosphorylation of EGFR and the downstream signaling in the triple-mutant EGFR PC-9 cells. Furthermore, intraperitoneal administration of 10 mg/kg lamellarin 14 for 17 days suppressed tumor growth of the triple-mutant

Abbreviations: CDK, cyclin-dependent kinase; del ex19, in-frame deletion within exon 19; DYRK, dual-specificity tyrosine-phosphorylation-regulated kinase; EGFR, epidermal growth factor receptor; GI_{50}, 50% growth inhibition; GSK, glycogen synthase kinase; IL, interleukin; Lam, lamellarin; LamN, lamellarin N; NSCLC, non-small-cell lung cancer; PIM, protooncogene serine/threonine-protein kinase; TKI, tyrosine kinase inhibitor; Topo, topoisomerase.

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EGFR PC-9 cells in a mouse xenograft model using BALB/c nu/nu mice. Thus, lamellarin 14 serves as a novel structural backbone for an EGFR-TKI that prevents the development of cross-resistance against known drugs in this class.

KEYWORDS

epidermal growth factor receptor, lung cancer, recurrence, topoisomerase inhibitor, tyrosine kinase inhibitor

1 | INTRODUCTION

Activating mutations within the region encoding the kinase domain of EGFR often lead to the development of NSCLCs. Frequently observed mutations are the single-base substitution L858R or del ex19. Most cells with these mutations are sensitive to EGFR-TKIs, which bind to the ATP-binding pocket of the kinase domain.[1-4] EGFR-TKIs have improved clinical outcomes in patients with EGFR mutation-positive NSCLC.[5-11] Despite the strong clinical efficacy of EGFR-TKIs, most of the patients experience recurrence within a few years. The gatekeeper mutation T790M in the EGFR kinase domain decreases the binding affinity of first-generation EGFR-TKIs, such as gefitinib and erlotinib, to the ATP-binding pocket.[12] Therefore, this mutation results in the development of resistance to EGFR-TKIs in 50% of the cases of EGFR-TKI-treated NSCLC.[13-16]

To overcome this limitation of the first-generation inhibitors, irreversible EGFR-TKIs that form a covalent bond with the thiol group of the Cys797 residue are now clinically available.[17-19] However, the estimated clinical utility of a second-generation TKI, afatinib (BIBW2992), was less than expected due to its dose-limiting toxicity associated with the concurrent inhibition of WT EGFR in normal tissues.[20] A third-generation EGFR-TKI, osimertinib (AZD9291), has a pyrimidine-base structure that shows much higher selectivity for the mutant EGFRs than the first- and second-generation TKIs that have anilinoquinazoline-based structures.[18,19] Osimertinib was originally approved as a second-line therapy for T790M mutation-positive cases of NSCLCs, and it has been recently considered as a first-line therapy because it improves overall survival better than gefitinib or erlotinib.[21] However, the efficacy of osimertinib can be again compromised by acquired resistance from various mechanisms.[22-28] The C797S substitution mutant EGFR lacks Cys797, the site for the formation of a covalent bond with the Michael acceptor group of osimertinib.[22,23] Currently available EGFR-TKIs are ineffective in treating patients with triple mutations, including those with del ex19/T790M/C797S or T790M/C797S/L858R. Several attempts for discovering EGFR-TKIs that do not rely on Cys797[29,30] have not yielded results that have been clinically approved.

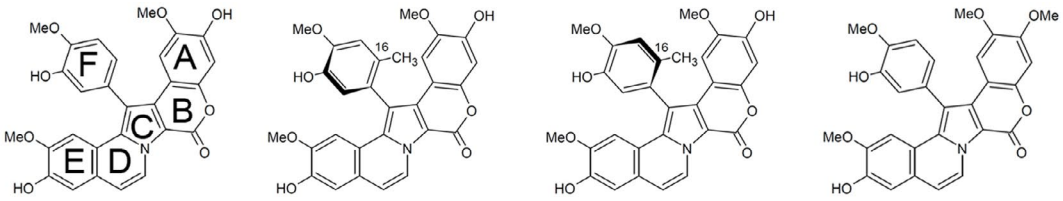
Lamellarins are natural marine alkaloids originally isolated from the prosobranch mollusk *Lamellaria* sp. They structurally belong to 3,4-dihydroxyphenylalanine (DOPA) derivatives.[31-33] Lamellarins show various biological activities, such as inhibitions of DNA topoisomerase I (Topo I),[34] HIV integrase,[35] and serine/threonine kinases, including cyclin-dependent kinases (CDKs) and glycogen synthase kinase (GSK)-3α/β, protooncogene serine/threonine-protein kinase (PIM)1, and dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK)1A.[36] The strong but nonselective kinase inhibitory activity of LamN can be harnessed by axial chirality-specific derivatization. One of the atropisomers of 16-methyllamellarin N, the (a*S*) form, is more selective for GSK-3, PIM1, and DYRKA1A than the (a*R*)-form.[37] Furthermore, both (a*R*) and (a*S*) 16-methyllamellarin N and the introduction of an additional methoxy group to the A-ring of LamN (20-O-methyllamellarin N, Lam3) caused the loss of their original biological activity as a Topo I inhibitor[37] (Figure 1). These findings suggest that the Lam scaffold is a unique structural motif that can be used to design selective inhibitors of protein kinases. By derivatizing a dual Topo I and protein kinase inhibitor, LamN, we generated a potent non-covalent EGFR-TKI, Lam14, that shows in vitro efficacy against the T790M gatekeeper mutation.[38]

In this study, we evaluated the biological activity of Lam14 in cells and in vivo. Lam14 inhibited the EGFR-mediated signaling and proliferation of cells expressing the osimertinib-resistant EGFR mutants del ex19/T790M/C797S or L858R//T790M/C797S. Moreover, Lam14 decreased the growth of triple-mutant EGFR PC-9 cells in vivo in a mouse xenograft model.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Ba/F3 cells[39] were provided by RIKEN BioResource Research Center and maintained in RPMI-1640 medium containing 10% FBS, 10 ng/mL recombinant mouse IL-3 (PeproTech), and penicillin/streptomycin. pBABE-EGFR L858R (#11012), pBABE-EGFR del exon19 (#32062), pBABE-EGFR L858R/T790M (#32073), and pBABE-EGFR del ex19/T790M (#32072) were obtained from Addgene. pBABE-EGFR L858R/T790M/C797S and pBABE-EGFR del ex19/T790M/C797S were constructed by site-directed mutagenesis with C797S-1 (GACATAGTCCAGGAGGCTGCCGAAGGGCATGAG) and C797S-2 (GACATAGTCCAGGAGTCAGCCGAAGGGCATGAG) primer set using pBABE-EGFR L858R/T790M or pBABE-EGFR del ex19/T790M as templates. pBABE-EGFR WT was also constructed by site-directed mutagenesis with R858L-1 (ACAGATTTTGGGCTGGCCAAACTGCTGGGT) and R858L-2 (ACCCAGCAGTTTGGCCAGCCCAAAATCTGT) primer set using pBABE-EGFR L858R as a template. Ba/F3 cells were transfected with plasmids (2 μg) using the Neon Transfection System (Thermo Fisher Scientific), according to the manufacturer's instructions. Stable



	lamellarin N	(aR)-16-methyllamellarin N	(aS)-16-methyllamellarin N	lamellarin 3
Topoisomerase I	+	-	-	-
CDK1 / cyclin B	++	++	-	++
CDK5 / p25	++	++	-	++
GSK-3α/β	+++	+	+	++
PIM1	++	+	+	++
DYRK1A	++	++	+	++

FIGURE 1 Derivatization of lamellarin N, a dual topoisomerase I (Topo I) and protein kinase inhibitor, restricts its target to specific kinases. Atropisomers of 16-methyllamellarin N, the (a*R*) and (a*S*) forms, and lamellarin 3 lose Topo I inhibitory activity. Furthermore, the (a*S*) form is more selective for glycogen synthase kinase (GSK)-3α/β, protooncogene serine/threonine-protein kinase (PIM)1, and dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK)A1A than the (a*R*)-form. Presence (+) or absence (−) of Topo I inhibition at 10 μM is indicated. IC_{50} values for protein kinases lower than 0.01, 0.1, and 1 are indicated as +++, ++, and +, respectively. Data from Baunbæk et al.[36] and Yoshida et al.[37]

transfectants were selected by 1 μg/mL puromycin (Nacalai Tesque) for 4 days, followed by removal of IL-3 for 1 week. Cells were cloned in 96-well plates by single-cell dilution. Ba/F3 stable transfectants were maintained without IL-3, except the one expressing WT EGFR. PC-9 cells were maintained in RPMI-1640 medium containing 10% FBS and penicillin/streptomycin. For the selection of stable EGFR transfectants, PC-9 cells were transfected with 2 μg plasmids using ViaFect Transfection Reagent (Promega). After 2 days, stable transfectants were selected using 2 μg/mL puromycin. Cells were cloned in 96-well plates by single-cell dilution. Osimertinib and gefitinib were purchased from Selleck Chemicals.

2.2 | JFCR39 panel assays and COMPARE analysis

The JFCR39 panel assays were developed to evaluate the antitumor profile of test compounds across a panel of 39 human cancer cell lines (termed JFCR39), as previously described.[40] To this end, the concentration of a test compound required for GI_{50} in each of the 39 cell lines was calculated. Among the cell lines used, MKN-A and MKN-B are the cell lines that we used to refer to as MKN28 and MKN7, respectively, because they were identified as the sublines of MKN74 after careful consideration of short tandem repeat analyses (unpublished observations, 2016) and renamed. Fingerprints are presented in the graphic profiles of relative values of logarithmic GI_{50} concentrations across the JFCR39.[40] COMPARE analysis was carried out to evaluate the mechanism of action by calculating the Pearson correlation coefficients between the fingerprint of a test compound and those of reference compounds, as previously described.[41,42]

2.3 | LinCAGE analysis

Human lung cancer PC-9 cells were left untreated or treated with 10 μM LamN, Lam3, or Lam14 for 6 hours. Posttreatment, the total RNA of each treatment sample was extracted using the RNeasy Mini Kit (Qiagen). Whole transcriptome data of the extracted RNAs were obtained using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix). To estimate the target molecular pathways of each compound, we undertook connectivity scoring analysis in the JFCR_LinCAGE database, which compared the gene expression signature of cells treated with the query compound with those of cells treated with a wide range of anticancer drugs or molecularly targeted agents in the database, as previously described.[43] For the analysis, we extracted and used the signature probe sets whose expression changes after each compound treatment were significantly up- or downregulated using Student's *t* test (fold change greater than 2 and a *P* value less than .05).

2.4 | Cell viability assay

Ba/F3 (3000 cells/well), PC-9 (3000 cells/well), or A549 cells (7500 cells/well) were seeded into 96-well plates. Interleukin-3 (10 ng/mL) was added to Ba/F3 cells when needed. Drugs were serially diluted,

added to cells, and incubated for 4 days. Thiazolyl blue tetrazolium bromide (0.5 mg/mL MTT) was added, and cells were incubated for 4 hours. The resultant formazan was solubilized with 10% SDS. The OD_{570} was measured by a microplate reader (Molecular Devices).

2.5 | Western blot analysis

Whole-cell extracts were prepared by lysing cells with RIPA buffer (50 mM Tris-HCl [7.4], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing 5 mM sodium vanadate, 5 mM NaF, and complete EDTA-free protease inhibitor (Roche) for 30 minutes at 4°C. Insoluble materials were removed by centrifugation. Western blotting was carried out using standard methods. The following antibodies were used: rabbit anti-phospho-EGFR (Y1068) (1:3000; Cell Signaling #2237), mouse anti-EGFR (1:3000; Cell Signaling #2239), rabbit anti-phospho-AKT (S473) (1:3000; Cell Signaling #9271), rabbit anti-AKT (1:3000; Cell Signaling #9272), rabbit anti-phospho-ERK1/2 (T202/Y204) (1:3000; Cell Signaling #9101), rabbit anti-ERK1/2 (1:10 000; Santa Cruz sc-93), mouse anti-actin (1:1000; Sigma C7207), anti-mouse IgG-HRP (1:10 000; GE Healthcare NA9310), and anti-rabbit IgG-HRP (1:10 000; GE Healthcare NA9340). Blots were developed using the ECL Western Blotting Detection Reagents (GE Healthcare RPN2109) and detected using the LAS-3000 imaging system (Fujifilm).

2.6 | Xenograft

The study was approved by the Animal Care Committee of Iwate Medical University (Approval ID: 27-029). Experiments were carried out in accordance with the Guidelines of the Iwate Medical University Ethics Committee for Animal Treatment and the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan. PC-9-EGFR-del exon19/T790M/C797S cells (5×10^6 cells) were suspended in 100 μL of 1:1 Matrigel and subcutaneously implanted into BALB/c nu/nu mice (Charles River) using a 21G syringe. Tumor growth was monitored every day by bilateral caliper measurement, and tumor volume (mm^3) was calculated as length (mm) × $width^2$ (mm) / 2. After 1 week, mice were intraperitoneally injected with PBS or 5 or 10 mg/kg Lam14 once a day for 17 days.

2.7 | Immunohistochemistry

The tumor xenografts were dissected from mice, fixed in 4% paraformaldehyde, and embedded in paraffin wax to produce 6-7-μm sections. After blocking with 5% normal horse serum, the sections were incubated with the anti-phospho-EGFR (Tyr1068) Ab (#2234, Cell Signaling Technology, 1:100) or the anti-EGF Ab (#2232, Cell Signaling Technology, 1:100). We used DAB (Vector Laboratories) as the chromogen. The sections were counterstained with hematoxylin. Images were analyzed using ImageJ.

3 | RESULTS

3.1 | Modifications in the A-ring of LamN converted the target orientation from Topo I to protein kinases in cells

Modifying the A-ring of LamN altered target orientation and inhibited the EGFR T790M/L858R mutant at a low nanomolar IC_{50} in vitro (8.9 nM).[38] To examine the target transition among lamellarin derivatives at the cellular level, two bioinformatic approaches were adopted. First, biological activities of the parental lamellarin (LamN), 20-O-methyllamellarin N (Lam3), and Lam14 across the JFCR39 cancer cell line panel were determined and the fingerprints were subjected to COMPARE analysis[40-42] (see Section 2; Figure 2). Because the fingerprint reflects the overall biological activity of the compound, a mechanism of action can be predicted based on the similarity with fingerprints of reference compounds with known mechanisms of action in the database. As expected, LamN showed strong similarity to SN38, a DNA Topo I inhibitor, with the highest Pearson correlation coefficient ($r = 0.77$; Table S1). In contrast, the Pearson correlation coefficient between Lam3 and SN38 was reduced to 0.53 (Table S2). Finally, Lam14 showed a Pearson coefficient of only 0.23. Instead, Lam14 showed Pearson coefficients that indicated similarities to various protein kinase inhibitors, including CDK inhibitors such as PHA793887 (0.65), AT-7519 (0.57), and SNS-032 (0.54), tyrosine kinase inhibitors such as alectinib (0.60), olmutinib (0.55), and herbimycin A (0.53), and aurora kinase inhibitors, such as danusertib (0.62) and SNS-314 (0.52) (Table S3). The results strongly suggested the conversion of the target orientation of Lam14 from Topo I to protein kinases.

Second, a genome-wide analysis of the transcriptome was carried out to evaluate the transition of the mechanisms of action among the Lams. We extracted signature genes that were up- or downregulated in PC-9 cells treated with LamN, Lam3, or Lam14 and compared them with those of known anticancer drug treatments in our JFCR LinCAGE database.[43,44] The gene signature of LamN showed a striking similarity with those of Topo I inhibitors. Lamellarin 3, in which the hydroxy group on the A-ring of LamN is replaced with a methoxy group, showed similarities with the signatures of EGFR-TKIs and Topo I inhibitors. Lam14, which has two dimethylaminopropyl groups, indicated an EGFR-TKI-like signature without showing Topo I inhibitor-like character (Figure 3).

3.2 | Lamellarin 14 suppressed viabilities of Ba/F3 cells expressing TKI-resistant mutant EGFRs del ex19/T790M or del ex19/T790M/C797S

To verify the effect of Lam14 on activated and resistant mutant EGFRs, we established Ba/F3 cells expressing del ex19/T790M or del ex19/T790M/C797S EGFR mutants. Although parental Ba/F3 cells depend on IL-3 for their survival, the cells expressing an exogenous activated kinase survive even in the absence of IL-3.[45] Because Ba/F3 cells expressing the EGFR mutants depend on exogenous

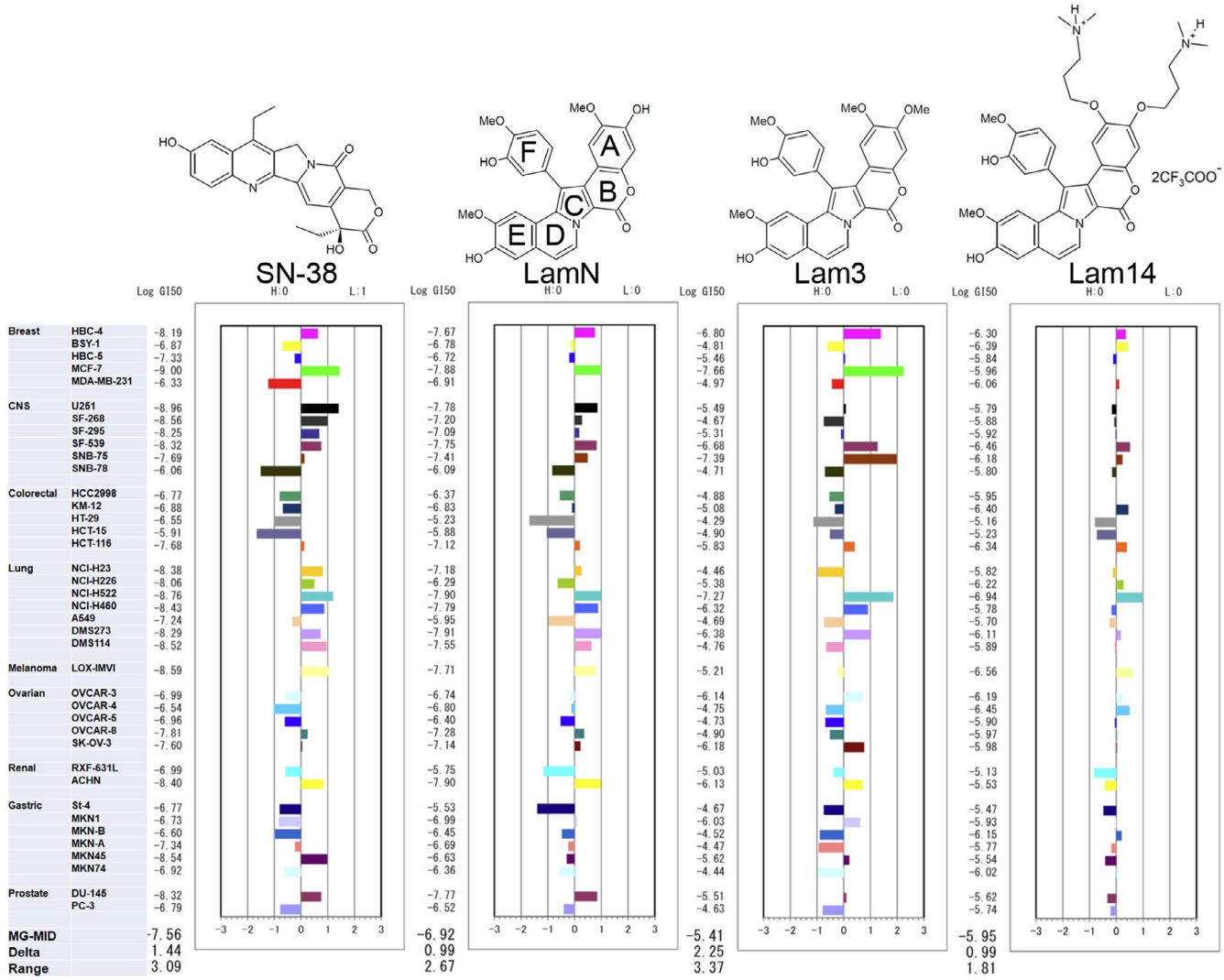


FIGURE 2 JFCR39 fingerprints reveal the transition of target orientations among lamellarin (Lam) derivatives with chemical modifications in the A-ring. JFCR39 fingerprints of a topoisomerase I inhibitor, SN38, and Lam derivatives show that LamN has the closest similarity to SN38. Furthermore, Lam14 lost its similarity with SN38. Each fingerprint is composed of the differential growth inhibition pattern of a test compound for the cancer cell lines in the JFCR39 panel. The X-axis indicates the difference from the mean of log 50% growth inhibition (GI_{50}) values for all 39 cell lines in the logarithmic scale (MG-MID, expressed as 0 in the fingerprint). The log GI_{50} values for each cell line are shown on the left. Structures of the test compounds are indicated on the top of the corresponding fingerprints. Columns to the right from 0 indicate sensitivity to a test compound; those to the left indicate resistance. MG-MID, mean of log GI_{50} values for all 39 cell lines; delta, the difference between the MG-MID and the log GI_{50} for the most sensitive cell line; range, the difference between the log GI_{50} values for the most resistant and most sensitive cell lines

EGFR activity instead of IL-3 signaling, EGFR inhibition opposes their survival in the absence of IL-3. Conversely, IL-3 treatment confers tolerance against EGFR inhibition in Ba/F3 cells. Therefore, growth suppression in the presence of IL-3 can be interpreted as nonspecific toxicity. Osimertinib and Lam14 suppressed the survival of Ba/F3 cells expressing EGFR del ex19/T790M at much lower concentrations under IL-3-depleted conditions than those in the presence of 10 ng/mL IL-3 (Figure 4A,B). However, the suppressive effect of osimertinib was decreased in Ba/F3 cells expressing EGFR del ex19/T790M/C797S, as previously described[23,30] (Figure 4A). In contrast to osimertinib, Lam14 maintained its efficacy against EGFR del ex19/T790M/C797S at a similar level to its inhibitory activity against EGFR del ex19/T790M (Figure 4B,C). Lam14 potently inhibits the activation of the mutant EGFR kinase in comparison WT EGFR kinase in vitro.[38] The Ba/F3 model also showed that mutant EGFR del ex19/T790M/C797S is more sensitive to Lam14 than WT EGFR (Figure 4C,D).

3.3 | Lamellarin 14 inhibited viabilities and EFGR signaling of NSCLC cell lines expressing EGFR mutants positive for TKI-resistant mutations T790M or T790M/C797S

Next, we verified the efficacy of these compounds in PC-9 NSCLC cells expressing exogenous EGFR mutants. A first-generation EGFR-TKI, gefitinib, was only effective in parental PC-9 cells expressing endogenous

(A)

LamN

rank	Cell	Compound	concentration	score
1	PC9	SN-38	3.00E-06	1
2	HT29	SN-38	3.00E-06	0.94422
3	Skov3	SN-38	3.00E-06	0.94078
4	K562	SN-38	1.00E-06	0.92805
5	HT29	Camptothecin	3.00E-06	0.91529
6	HT29	Melphalan	1.00E-04	0.89595
7	HT29	Actinomycin D	3.00E-08	0.8598
8	HT29	Topotecan	3.00E-06	0.84114
9	HT29	Cisplatin	3.00E-05	0.82159
10	H2228	SN38	3.00E-06	0.76645
11	HT29	Nimustine	0.001	0.72003
12	HT29	SB218078	3.00E-06	0.6926
13	HT29	NU6102	3.00E-05	0.68181
14	HT29	Mitoxantrone	3.00E-06	0.6724
15	HT29	Ruxolitinib	1.00E-04	0.67173
16	HT29	Neocarzinostat	3.00E-06	0.66156
17	HT29	Doxorubicin	3.00E-06	0.65996
18	PC9	Gefitinib30	3.00E-05	0.59483
19	HT29	Mitomycin C	1.00E-05	0.59226
20	HT29	PP242	1.00E-05	0.5875

Lam3

rank	Cell	Compound	concentration	score
1	PC9	Erlotinib0.6	6.00E-07	1
2	PC9	Erlotinib30	3.00E-05	0.99546
3	PC9	Gefitinib0.6	6.00E-07	0.99424
4	PC9	Gefitinib30	3.00E-05	0.97633
5	PC9	Afatinib	3.00E-08	0.93382
6	PC9	SN-38	3.00E-06	0.88767
7	HT29	PP242	1.00E-05	0.88078
8	HT29	Sunitinib	1.00E-05	0.88016
9	HT29	Sunitinib	1.00E-05	0.87103
10	HT29	Vemurafenib	3.00E-05	0.85082
11	HT29	PP242	1.00E-05	0.83832
12	HT29	Melphalan	1.00E-04	0.82517
13	HT29	SU11274	3.00E-05	0.8195
14	HT29	Cabozantinib	3.00E-05	0.81313
15	HT29	U-0126	3.00E-05	0.79091
16	HT29	SN-38	3.00E-06	0.7777
17	HT29	Topotecan	3.00E-06	0.77003
18	HT29	Actinomycin D	3.00E-08	0.75903
19	K562	SN-38	1.00E-06	0.75885
20	HT29	Cisplatin	3.00E-05	0.75465

Lam14

rank	Cell	Compound	concentration	score
1	PC9	Erlotinib30	3.00E-05	1
2	PC9	Erlotinib0.6	6.00E-07	0.9715
3	PC9	Afatinib	3.00E-08	0.96606
4	PC9	Gefitinib30	3.00E-05	0.96337
5	PC9	Gefitinib0.6	6.00E-07	0.95687
6	HT29	Sunitinib	1.00E-05	0.86838
7	HT29	Pazopanib	3.00E-05	0.86309
8	HT29	PP242	1.00E-05	0.86063
9	HT29	PP242	1.00E-05	0.83975
10	HT29	Vemurafenib	3.00E-05	0.82537
11	HT29	Sunitinib	1.00E-05	0.81167
12	HT29	SU11274	3.00E-05	0.79831
13	HT29	Cabozantinib	3.00E-05	0.79006
14	HT29	6-Mercaptopur	1.00E-04	0.77815
15	HT29	U-0126	3.00E-05	0.77498
16	PC9	Trametinib	1.00E-06	0.77098
17	HT29	NU6102	3.00E-05	0.76655
18	H2228	Crizotinib	1.00E-05	0.76365
19	HT29	Cdk4 inhibitor	1.00E-05	0.76205
20	HT29	BEZ235	1.00E-06	0.74916

Topoisomarase inhibitors

EGFR-TKI

(B)

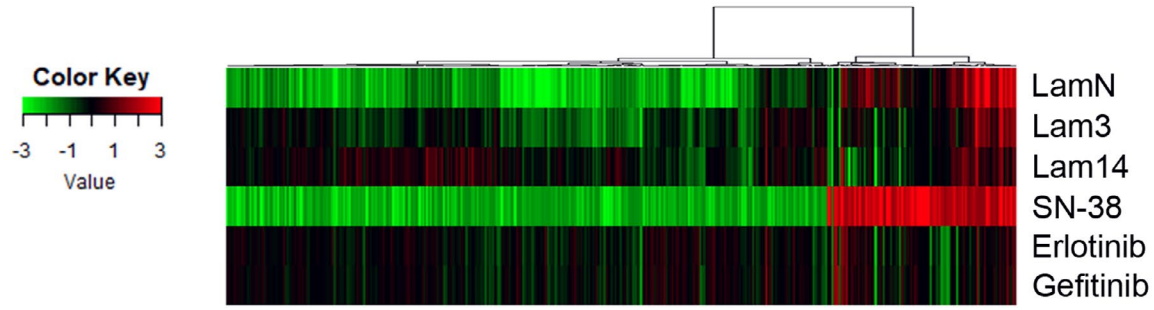


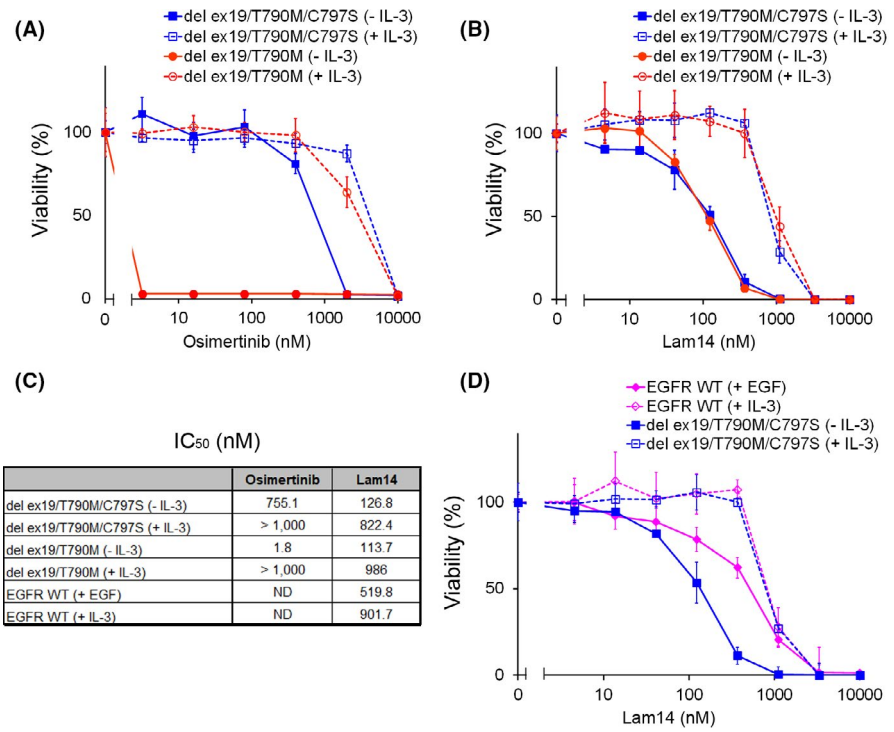
FIGURE 3 Genome-wide transcriptomic analysis reveals the transition of the mechanisms of action among the lamellarins (Lams). A, Total RNAs extracted from PC-9 cells treated with 10 μM LamN, Lam3, or Lam14 for 6 h were subjected to microarray analysis and compared with gene expression patterns (signatures) of known anticancer drug treatments in the JFCR LinCAGE database. For the analysis, we extracted and used the signature probe sets whose expression was significantly up- or downregulated using Student's *t* test (fold change >2 and $P < .05$). The top 20 compounds whose signatures showed significant similarities to those of LamN, Lam3, or Lam14 are listed. B, Hierarchical clustering analysis based on the gene set whose expression was significantly up- or downregulated in SN38-treated PC-9 cells (fold change >3) is shown. Values in the heat map are the logarithmic values of the sample-to-control ratio of intensity values. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor

EGFR del ex19 (Figure 5A). Osimertinib reduced the proliferation of PC-9 cells expressing endogenous EGFR del ex19 and exogenous EGFRs del ex19/T790M or L858R/T790M. However, it did not show efficacy in PC-9 cells expressing exogenous del ex19/T790M/C797S or L858R/T790M/C797S (Figure 5B). Conversely, Lam14 suppressed the proliferation of all PC-9 cells expressing each exogenous EGFR mutant regardless of the existence of osimertinib-resistant C797S mutation at similar dose ranges (Figure 5C,D). A549 cells expressing endogenous WT EGFR were insensitive (Figure 5C), consistent with in vitro kinase inhibition[38] and the Ba/F3 system (Figure 4C).

The effects of Lam14 on the intracellular EGFR signaling were analyzed by monitoring the phosphorylation status of EGFR, ERK, and AKT in PC-9 cells expressing EGFR mutants. Lam14 diminished the levels of phosphorylated EGFR and downstream signaling regardless of the type of EGFR mutations in the same dose range, whereas osimertinib lost its inhibitory activities in the C797S mutant cells (Figure 5E,F).

3.4 | Lamellarin 14 suppressed tumor growth of PC-9 cells expressing EGFR del ex19/T790M/C797S in vivo

The antitumor effects of Lam14 were analyzed in mouse xenograft models using PC-9 cells expressing EGFR del ex19/T790M/C797S. The cells were subcutaneously transplanted into BALB/c nu/nu



	Osimertinib	Lam14
del ex19/T790M/C797S (- IL-3)	755.1	126.8
del ex19/T790M/C797S (+ IL-3)	> 1,000	822.4
del ex19/T790M (- IL-3)	1.8	113.7
del ex19/T790M (+ IL-3)	> 1,000	986
EGFR WT (+ EGF)	ND	519.8
EGFR WT (+ IL-3)	ND	901.7

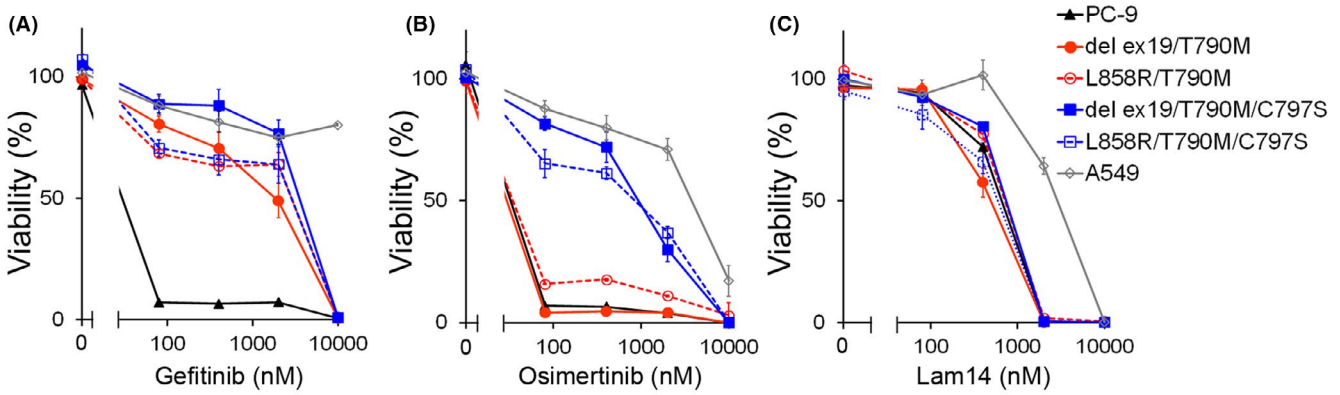
FIGURE 4 Lamellarin (Lam)14 reduces the viability of Ba/F3 cells expressing epidermal growth factor receptors (EGFRs) with in-frame deletion within exon 19 (del ex19)/T790M or del ex19/T790M/C797S EGFR-tyrosine kinase inhibitor-resistant mutations. A-C, MTT assay was used to determine the viability of Ba/F3 cells expressing WT or mutant EGFRs treated with Lam14 or osimertinib for 4 days in the presence or absence of interleukin-3 (IL-3; 10 ng/mL). Ba/F3 cells expressing del ex19/T790M or del ex19/T790M/C797S EGFR mutations were treated with the indicated concentration of osimertinib (A) or Lam14 (B) in the presence or absence of IL-3 (10 ng/mL) (mean ± SD [n ≥ 3]). C, IC_{50} values of osimertinib and Lam14 in Ba/F3 cell lines are indicated. ND, not determined. D, Ba/F3 cells expressing WT EGFR or Ba/F3 del ex19/T790M/C797S cells were treated with the indicated concentration of Lam14 in the presence or absence of IL-3 (10 ng/mL) (mean ± SD [n = 3]). In Ba/F3 WT cells, EGF (10 ng/mL) was added instead of IL-3

mice, and Lam14 was intraperitoneally injected at 5 or 10 mg/kg. Treatment with 10 mg/kg Lam14 significantly inhibited the growth of EGFR del ex19/T790M/C797S tumors in vivo. Treatment with 5 mg/kg Lam14 tended to suppress the growth, but this decrease was not statistically significant (Figure 6A). Of note, treatment with the effective dose of Lam14 neither caused weight loss in comparison with the control nor yielded obvious toxicity (Figure 6B). Moreover, Lam14 decreased the level of EGFR phosphorylation in the del ex19/T790M/C797S xenografts (Figure 6C,D).

4 | DISCUSSION

In this study, we found that the derivatization of a marine natural product, LamN, altered its molecular target from Topo I to protein kinases. Modification in the A-ring of LamN created a novel class of EGFR-TKI, Lam14, that overcame the acquired resistance to third-generation EGFR-TKIs. Lam14 diminished the viability of Ba/F3 and PC-9 cells expressing EGFR del ex19/T790M/C797S triple-mutant in addition to cells expressing EGFR del ex19 or del ex19/T790M. Lam14 inhibited EGFR signaling in PC-9 cells expressing the triple mutants. Moreover, Lam14 suppressed tumor growth of the triple-mutant EGFR PC-9 cells in vivo. Thus, Lam14 is a promising seed compound for overcoming the acquired resistance to third-generation EGFR-TKIs. Further structural alterations in Lams could confer innovative structural backbones for EGFR-TKIs, which avoid cross-resistance to known EGFR-TKIs.

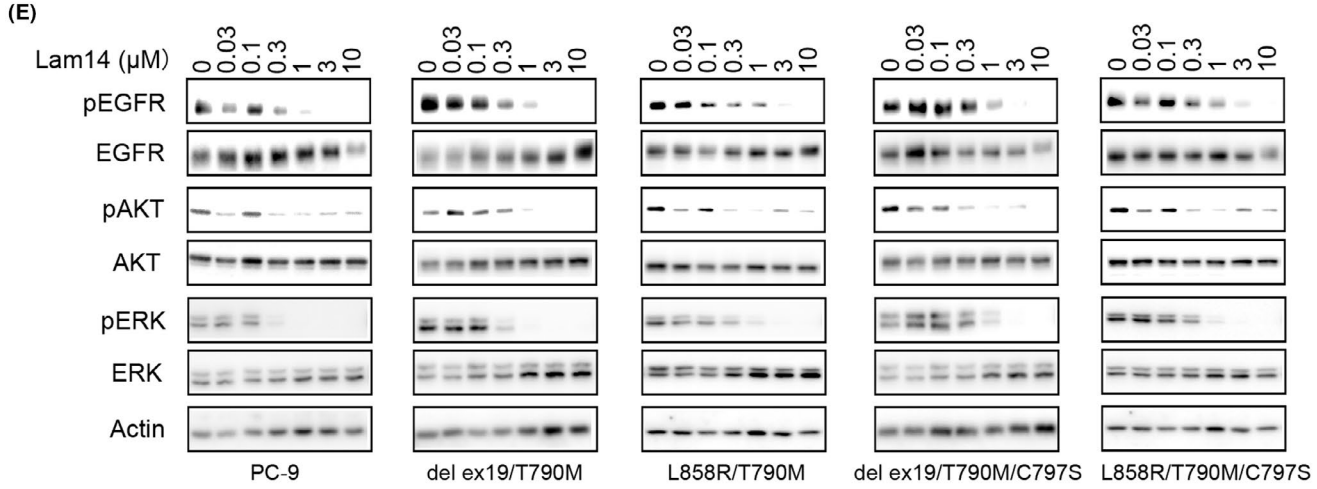
Derivatization of LamN transformed a marine natural Topo I inhibitor into a protein kinase inhibitor belonging to a new structural class. Bioinformatic analyses, the JFCR39 panel fingerprints, and LinCAGE analysis revealed that the introduction of two dimethylaminopropyl groups to the A-ring of the lamellarin structure converted the Topo I inhibitor-like profile of LamN into a protein kinase inhibitor-like profile (Figures 2 and 3). Lam14 inhibits EGFR kinase in vitro at a low nanomolar order.[38] In this study, Lam14 inhibited mutant EGFR-dependent signaling (Figures 5E and 6C,D), cell proliferation (Figures 4B and 5C), and tumor growth in vivo (Figure 6A). In addition to inhibiting Topo I and EGFR kinase, Lams have shown inhibitory activity against HIV integrase[35] and serine/threonine kinases, including CDKs and GSK-3α/β, PIM1, and DYRK1A.[36] Although we focused on the inhibition of EGFR mutants in this study, the target specificity of Lam14 needs to be examined. Nonbiased gene signature-based LinCAGE analysis classifies kinase inhibitors based on their target kinase pathways,[43] and this analysis revealed a specific similarity between Lam14 and particular kinase inhibitors (Figure 3A,B). The JFCR39 panel fingerprints also pointed out the



(D)

IC_{50} (nM)

	Gefitinib	Osimertinib	Lam14
PC-9	1.3	< 1	658.5
del ex19/T790M	> 1,000	< 1	493.9
L858R/T790M	> 1,000	< 1	719
del ex19/T790M/C797S	> 1,000	927.3	739.9
L858R/T790M/C797S	> 1,000	844.9	595.7
A549	> 1,000	> 1,000	> 1,000



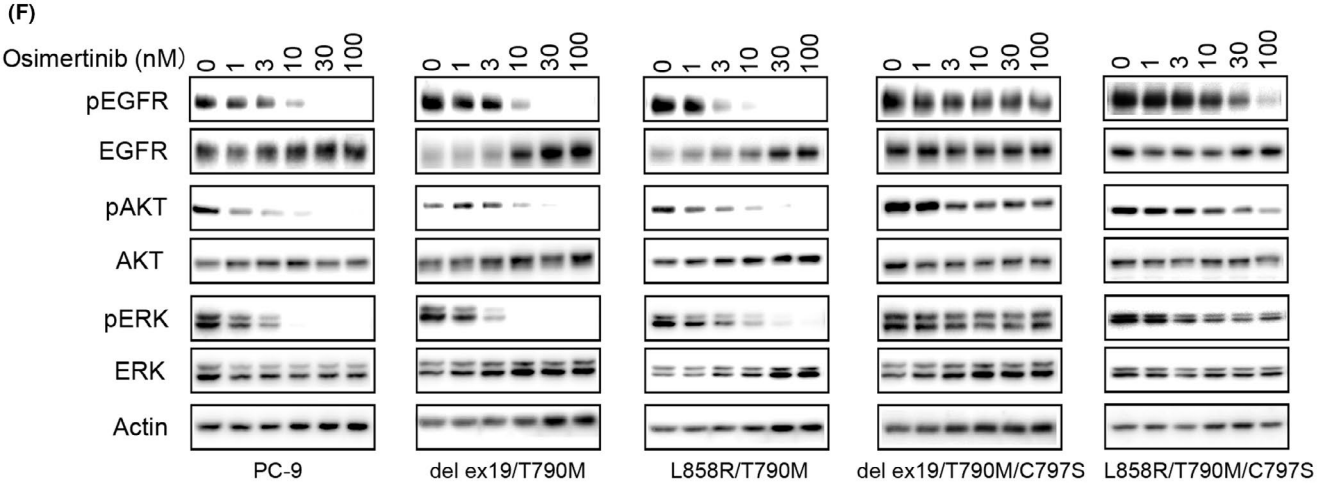


FIGURE 5 Lamellarin (Lam)14 inhibits cell viability and epidermal growth factor receptor (EGFR) signaling in PC-9 cells expressing EGFR-tyrosine kinase inhibitor-resistant mutant EGFRs. A–C, Parental PC-9 cells, mutant EGFR-expressing PC-9 cells (in-frame deletion within exon 19 [del ex19]/T790M, L858R/T790M, del ex19/T790M/C797S, L858R/T790M/C797S), and A549 cells expressing endogenous WT EGFR were treated with the indicated concentration of gefitinib (A), osimertinib (B), or Lam14 (C) for 4 days (mean ± SD [n = 3]). Cell viability was measured using the MTT assay. D, IC_{50} values of gefitinib, osimertinib, and Lam14 in PC-9 cell lines are indicated. E, F, PC-9 cells and mutant EGFR-expressing PC-9 cells (del ex19/T790M, L858R/T790M, del ex19/T790M/C797S, or L858R/T790M/C797S) were treated with Lam14 (E) or osimertinib (F) for 4 hours. Phosphorylation levels of EGFR and its downstream molecules were analyzed by western blotting using Abs for indicated proteins. pAKT, AKT phosphorylated at S473; pEGFR, EGFR phosphorylated at Y1068; pERK, ERK1/2 phosphorylated at T202/Y204

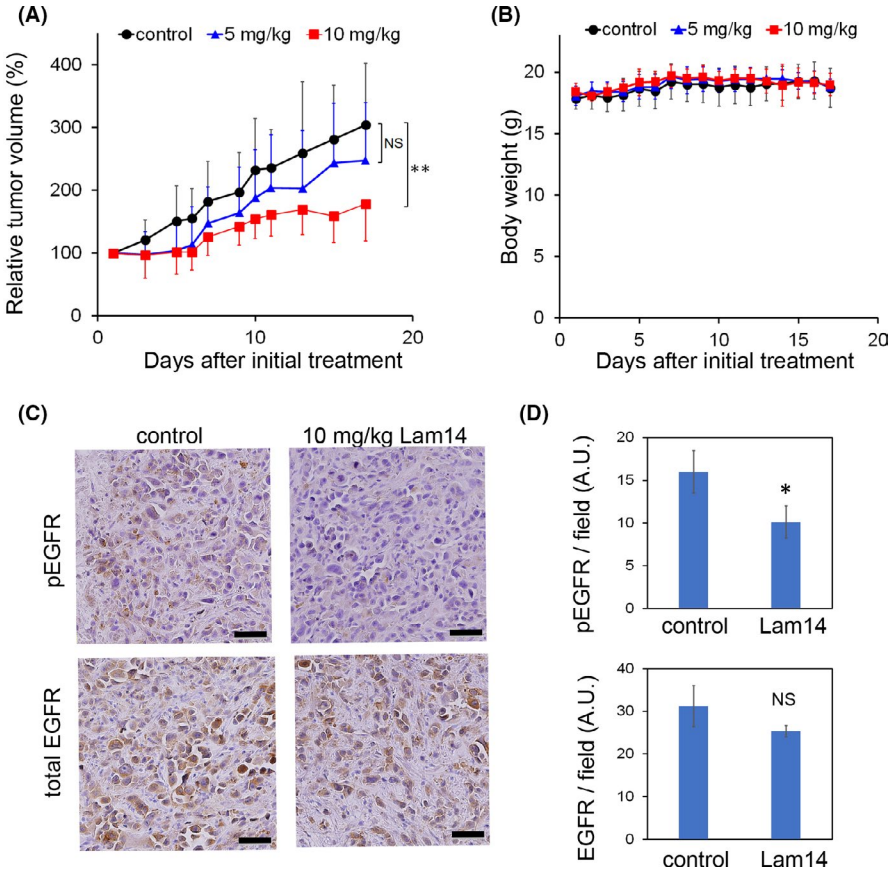


FIGURE 6 Lamellarin (Lam)14 suppresses tumor growth and epidermal growth factor receptor (EGFR) phosphorylation in EGFR in-frame deletion within exon 19 (del ex19)/T790M/C797S-expressing PC-9 cells in vivo. A, PC-9 del ex19/T790M/C797S cells were s.c. injected into BALB/c nu/nu mice. One week after the implantation, the mice were randomized into control (PBS) or treatment groups (5 or 10 mg/kg Lam14). PBS or Lam14 was i.p. injected once a day for 17 days. Tumor volume (mm^3) was calculated as length × $width^2$ / 2 (mean ± SD [n ≥ 9]). Change in tumor size on day 17 was calculated using one-way ANOVA. NS, not significant. $^{**}P < .01$. B, Body weights of mice were measured every day (mean ± SD [n = 5]). C, D, Levels of EGFR phosphorylation in tumors were analyzed by immunohistochemistry (C), and at least eight independent view fields for each of the four tumors were analyzed using ImageJ and Student's *t* test. NS, not significant. $^{*}P < .05$. Scale bars = 50 μm

similarities between Lam14 and olmutinib, a third-generation EGFR-TKI that is effective against the EGFR T790M mutant (Table S3). These data suggest that Lam14 inhibits mutant EGFRs with substantial specificity. Alectinib is an ALK-TKI that has a rigid and a planar structure (Figure 7), and it is effective for treating crizotinib-resistant ALK mutants, such as the gatekeeper mutant EML4-ALK L1196M.[46] Of note, as per COMPARE analysis, Lam14's fingerprint showed a moderate but significant correlation with that of alectinib ($r = 0.60$, $P < 5.5 \times 10^{-5}$; Table S3). Topoisomerase inhibitors, which generally possess a planar structure (Figure 7), can be transformed into new structures that bind to the target kinases differently from known kinase inhibitors.

Lam14 is effective against EGFR mutants, including the third-generation EGFR-TKI-resistant C797S. Ba/F3 and PC-9 cells expressing the third-generation EGFR-TKI-resistant del ex19/T790M/C797S triple-mutant were sensitive to the same dose range of Lam14 as the effective dose for cells expressing EGFR del ex19 or del ex19/T790M (Figures 4B and 5C). Lam14 also decreased the autophosphorylation of EGFR and the downstream signaling in the triple-mutant EGFR-expressing PC-9 cells (Figure 5E). Furthermore, Lam14 suppressed the in vivo growth of the triple-mutant EGFR PC-9 cells in a mouse xenograft model (Figure 6A). Although brigatinib[30] and the allosteric EGFR-TKI inhibitor EAI045[29] (Figure 7) have been reported to be effective against the triple-mutant in vivo at 75 and 60 mg/kg, respectively, Lam14 showed tumor growth inhibition that is comparable with those of such compounds at 10 mg/kg without causing weight loss and obvious toxicity (Figure 6). Thus, Lam14 could overcome drug-resistant EGFR mutations. New structural classes of TKIs could prevent the development of cross-resistance with known TKIs containing quinazoline- or pyrimidine-based structures.

Lam14 preferentially inhibits the proliferation of cells expressing mutant EGFRs compared with cells expressing WT EGFR or cells dependent on other driver genes. Ba/F3 cells expressing TKI-resistant EGFRs were more sensitive to Lam14 than those

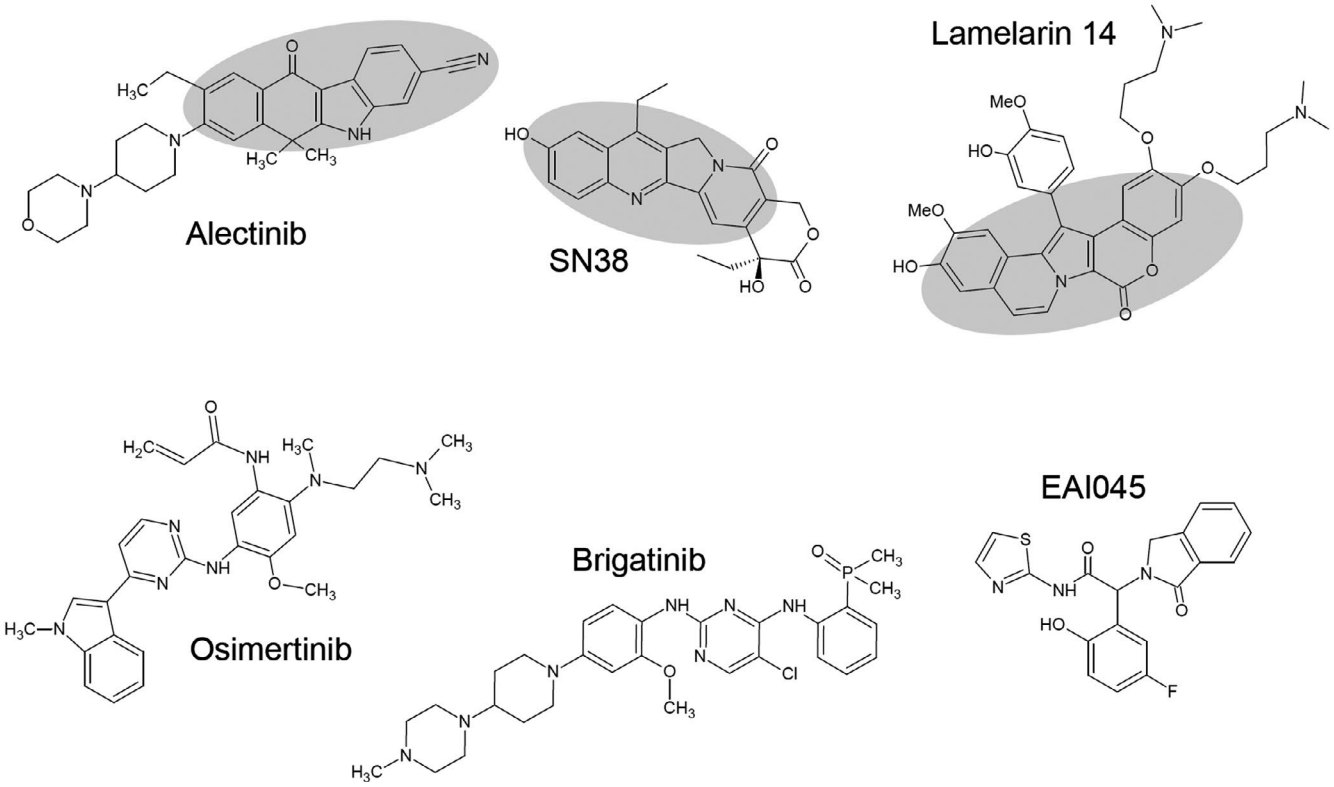


FIGURE 7 Chemical structures of SN38 and protein kinase inhibitors. Planar structures are shaded

expressing WT EGFR. In the absence of IL-3, IC_{50} values of cells expressing del ex19/T790M, del ex19/T790M/C797S, and WT EGFR were 113.7, 126.8, and 519.8 μM, respectively (Figure 4B-D), indicating that Lam14 was 4.1-5.6-fold more potent against cells expressing the EGFR-TKI resistant mutants than those expressing WT EGFR. Although the JFCR39 panel did not include cell lines harboring activating or TKI-resistant EGFR mutations, the existing EGFR-TKIs, including gefitinib, erlotinib, osimertinib, and olmutinib, showed a selective antitumor effect on lung cancer NCI-H522 and ovarian cancer SK-OV3 cells (Figure S1). SK-OV3 cells overexpress ErbB2 and their growth depends on ErbB2.[47,48] Therefore, the antitumor effects of typical EGFR-TKIs on SK-OV3 are likely due to their selective inhibition of ErbB2. In contrast, Lam14 did not exert a selective antitumor effect on SK-OV3 (Log GI_{50} −5.98; approximately 1.1 μM, Figure 2), suggesting that Lam14 does not appear to inhibit ErbB2 in SK-OV3 cells effectively. Likewise, brigatinib, another TKI effective on del ex19/T790M/C797S EGFR mutant cells, as mentioned above, exerted a modest effect on SK-OV3 cells (Fig. S1). Therefore, brigatinib likely has a limited effect on ErbB2 as well. However, COMPARE analysis revealed that brigatinib did not show a similar fingerprint to Lam14 ($r = 0.13$). Thus, the two compounds likely have different antitumor effects. In addition, the JFCR39 panel includes cell lines whose proliferation is addicted to the aberrant activation of a specific kinase, such as MET amplification in MKN45 cells and BRAF V600E mutation in HT-29 cells. However, none of these cells showed sensitivity to Lam14 (Figure 2). These data suggest Lam14's modest effects on MET and BRAF mutant cells.

The clinical efficacy of Lam14 or its derivatives need to be determined; however, a precise preclinical analysis must be undertaken first. Resistance to Lam14 might emerge from an unpredictable mechanism, similar to those of other TKIs.[22-28] Studying possible mechanisms of resistance to Lam14 could lead to the discovery of new resistant mutations and/or identify a common mechanism of resistance among structurally different classes of TKIs.

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DISCLOSURE

There are no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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