# Exploring Secondary Metabolites from Extremophilic Streptomyces for RET Inhibition in Non-Small Cell Lung Cancer

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***Abstract***

*Lung cancer is the second most prevalent cancer globally, with significant mortality rates among both smokers and nonsmokers. Targeting RET gene, which has been linked to the onset of non-small cell lung cancer (NSCLC) cases, offers a novel therapeutic avenue. Vandetanib, a tyrosine kinase inhibitor (TKI) of the RET gene, has several adverse effects, including skin toxicity, EGFR-induced diarrhea and VEGFR-related hypertension. Thus, we sought to identify promising candidates for RET inhibition employing a combination of in silico techniques. In this regard, we intend to investigate secondary metabolites from Streptomyces species, which are renowned for their therapeutic potential. A total of 3383 compounds produced by the Streptomyces species under extreme conditions were identified utilizing the antiSMASH database. Through molecular docking, the compounds were screened against the RET protein, identifying 43 compounds with better docking scores than vandetanib (-8.7 kcal/mol).*

*Subsequent ADMET analysis and machine learning validation using KDEEP, GNINA and RF scoring functions highlighted Esmeraldin B as a leading candidate. Esmeraldin B demonstrated superior binding affinity and interaction profiles, establishing five hydrogen bonds and hydrophobic interactions. Further, the scaffold analysis revealed that Esmeraldin B's phenazine moiety contributes to its potent antitumor properties. Collectively, these findings suggest that Esmeraldin B holds promise as a more effective and potentially safer alternative to vandetanib for RET-positive NSCLC. However, extensive in vitro and in vivo studies are required to confirm its therapeutic potential and to ensure safety for clinical and therapeutic applications.*

**Keywords:** Non-small cell lung cancer, REarranged during Transfection, Tyrosine kinase inhibitors, ML-SF, ADMET, Molecular Docking.

## Introduction

Lung cancer is the second most common cancer worldwide, with the highest incidence. While smoking accounts for over 80% of cases, lung cancer remains a top cause of cancer related deaths among non-smokers[16]. About 2% of patients have REarranged during Transfection (RET) mutations, primarily in non-small cell lung cancer (NSCLC), often with minimal smoking history and younger age[7,12]. The RET gene has at least 26 mutations that can cause illnesses, including multiple endocrine neoplasia type 2 (MEN2), which may result from activating point mutations[22]. There is a substantial correlation between the site of the point mutation and the disease phenotype.

Chromosome rearrangements can also cause constitutive activation of the RET kinase, leading to fusion genes, which are mostly linked to NSCLC[36]. Adding chemotherapy to surgery as adjuvant or neoadjuvant treatment can improve survival rates by roughly 5% at 5 years. However, these are generally not as specific as targeted therapies[15]. Targeted therapies against RET proteins have shown improved outcomes, including increased response rates and prolonged progression-free survival for patients with RET-positive NSCLC[1,12].

Multiple Kinase Inhibitors (MKIs) like cabozantinib and vandetanib initially offered hope for treating RET-positive NSCLC patients. Vandetanib functions as a treatment for RET rearranged lung cancer by targeting the abnormal activity of RET kinase that leads to cancer[41]. It inhibits ATP-binding sites, disrupting downstream signaling pathways for uncontrolled cell growth, survival and metastasis. When vandetanib was administered along with appropriate regimens of chemotherapy, patients exhibited better response[14]. Unfortunately, these nonselective MKIs showed limited durability in responses and caused off-target side effects such as skin toxicity and diarrhea and posed challenges for patient adherence in NSCLC[32]. Thus, alternative therapies are needed to counteract this issue.

This study aims to address the limitations of current drugs by investigating new generations of RET inhibitors, particularly medicinal compounds from bacteria in extreme environments with potent anti-cancer effects against the mutated RET gene. In this context, *in silico* approaches play a crucial role, allowing us to screen vast libraries of bacterial compounds rapidly and efficiently[20]. These computational techniques facilitate the identification of potential RET inhibitors by predicting their binding affinities and specificities. Additionally, it can identify the interactions between bacterial compounds and the RET protein, providing insights into their mechanisms of action[4]. These approaches help to prioritize compounds for experimental validation and to optimize lead compounds by predicting

their pharmacokinetic and pharmacodynamic properties, thereby enhancing the development of new RET inhibitors.

## Material and Methods

**Dataset Retrieval:** Our investigation into a potential drug compound targeting the 2IVU protein, commenced by utilizing the antiSMASH Version 7.0 database[6]. antiSMASH is the first all-encompassing pipeline that detects biosynthetic loci across a wide array of secondary metabolite classes such as polyketides, non-ribosomal peptides and many others. Our study focused predominantly on secondary metabolites produced by Streptomyces species thriving in challenging environments. Specifically, *Streptomyces sp. A144, Streptomyces sp. KY75, Streptomyces sp. INR7, Streptomyces sp. Mg1, Streptomyces huasconensis and Streptomyces leeuwenhoekii* were the target species. Their respective NCBI sequences were provided as input data and detection strictness was maintained at a rigorous level.

Additional features, including MIGB Comparison and Cluster Blast, were enabled before submission. The result obtained comprised of the whole genome sequence of the species from which the secondary metabolites produced by each gene were identified. For each identified secondary metabolite, detailed information concerning compound structure, PubChem ID, NPAtlas ID and the presence of 2D or 3D structures were systematically collected and documented.

**Protein preparation and Molecular Docking:** The protein structure was obtained from the Protein Data Bank (PDB ID: 2IVU) and it was prepared using BIOVIA Discovery Studio. Water molecules and heteroatoms were removed and polar hydrogen atoms were added. The three-dimensional structure of the reference compound vandetanib (ID - 3081361) was retrieved from PubChem in structural data format[19].The structures of the prepared 2IVU protein and the compounds to be docked were first loaded onto PyRx software, where molecular docking was performed using the AutoDock Vina mode to identify potential binding sites and ligand energies[9]. The parameters such as grid size and scoring functions were adjusted. The docking simulation was initiated to generate possible binding conformations out of which the most stable structure was selected for analysis. Compounds with docking scores more negative than vandetanib were selected for further analysis[21].

**ADMET analysis:** Pharmacokinetic parameters which control a drug's capacity to reach target proteins in the body and how long it stays in the bloodstream, are included in ADMET (absorption, distribution, metabolism, elimination and toxicity) analysis[26,30]. ADMET procedures are now commonly used in early-stage drug discovery to reduce attrition rates, emphasizing the importance of addressing these factors upfront to enhance drug efficacy and safety profiles. ADMET Lab 2.0 simplifies this process by providing comprehensive analysis of these properties[39]. The screened compounds were subjected to ADMET analysis to analyze the physicochemical properties of these compounds.The parameters including MW, nHA, nHD, LogP, HIA, F20%, F30%, PBB, BBB, CL, carcinogenicity, hERG blocker, rat oral toxicity were evaluated.

**Validation of results using Machine Learning Scoring Functions:** In recent years, machine-learning scoring functions (ML-SFs) based on protein-ligand complexes have shown substantial promise in specialized small-scale investigations[5]. To validate the docking results, three prominent ML-SFs such as KDeep, GNINA and RF Score were utilised. KDeep is a fast machine-learning approach for predicting binding affinities using State-of-the-Art 3D-convolutional neural networks[18]. GNINA utilizes an ensemble of convolutional neural networks (CNNs) as a scoring function. A CNN scoring function automatically learns the key features of protein-ligand interactions that correlates with binding[23]. RF score calculates the binding affinity of a protein-ligand pair in a complex[38]. Higher RF score value indicates better binding of the ligand into the binding site of the protein.

**Interaction analysis and anticancer activity-prediction:** In the drug discovery process, interaction analysis plays a pivotal role in understanding complex biological processes and mechanisms[3]. The docked configurations of each compound in PDB format were uploaded to PLIP for analysis[2]. PLIP provides visual representation of the protein-ligand complex, the interaction chain and different interaction types between each compound and the target protein. After perfoming PLIP analysis, the PASS server was used to predict the biological spectrum of the screened compounds. Using Bayesian estimates, the server predicts the likelihood of actives (Pa) and inactives (Pi) to differentiate the biological activities of hit compounds[13].

## Results and Discussion

**Mining Streptomyces genomes for anti-cancer metabolites:** antiSMASH7.0 is a renowned online genome mining tool used for predicting secondary metabolites synthesized by bacteria. The primary types of gene clusters involved in this biosynthesis include non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpenes, siderophores, lanthipeptides and ribosomally synthesized and post-translationally modified peptides (RiPP-like). Previous studies have indicated that NRPS, PKS and terpene gene clusters are among the most abundant, containing a vast array of bioactive compounds with varied functions including plant protection[11,28].

In total, 73 types of biosynthetic gene clusters (BGCs) were identified in the secondary metabolites produced by various Streptomyces species. It is important to note that organisms with multiple open reading frames (ORFs) typically encode a greater number of gene cluster regions[24]. The genome analysis of all *Streptomyces spp.* produced a total of 3383 putative secondary metabolites.

**Molecular docking and selection of candidate compounds:** Molecular docking aids in the identification of potential drug candidates by predicting the binding affinity of small molecules to a specific protein or receptor[31]. The RET-based protein 2IVU was prepared and docked with compounds after energy minimization using PyRx. Vandetanib was also docked against the target protein, yielding a docking score of -8.7 kcal/mol. The docking scores of the ligands were compared to this value, selecting those with scores lower (more negative) than vandetanib for further investigation. As shown in table 1, based on the threshold prescribed, out of 3383 compounds, 40 compounds were found to have lower docking scores in the range of -8.700 kcal/mol to -55.700 kcal/mol than vandetanib.

**Table 1**
**Docking and ML-SF scores of the reference and screened lead compounds.**

| S.N. | Compound name | Compound ID | Docking Score | ML-SF | | |
|---|---|---|---|---|---|---|
| | | | | KDeep | GNINA | RF |
| | | | | dG (kcal/mol) | CNN affinity | pK units |
| 1 | Vandetanib | 3081361 | **-8.7** | -11.386 | 7.202 | 6.025 |
| 2 | Urdamycin E | 175975 | **-8.7** | -9.805 | 6.718 | **6.209** |
| 3 | Kinamycin | 161863 | **-8.7** | -8.776 | 6.707 | 5.989 |
| 4 | Cyanogramide | 102143687 | **-8.9** | -7.07 | 5.611 | 5.95 |
| 5 | AQ 256 | 12435249 | **-8.9** | -7.2 | 4.932 | 5.971 |
| 6 | A879369 | 135957253 | **-9** | -7.466 | 5.176 | 5.955 |
| 7 | Dehydroxynocardamine | 11606728 | **-9** | -6.636 | 6.17 | 5.961 |
| 8 | Aurachin A | 6439172 | **-9.1** | -8.558 | 6.261 | 5.966 |
| 9 | Splenocin C | 42626285 | **-9.1** | -8.654 | 6.532 | **6.028** |
| 10 | Curamycin A | 71587265 | **-9.1** | **-15.996** | 6.412 | **7.093** |
| 11 | Neoantimycin | 585842 | **-9.1** | -9.451 | 6.541 | **7.093** |
| 12 | Frontalamide B | 101515038 | **-9.1** | -7.438 | 5.337 | 5.969 |
| 13 | Nybomycin | 169159 | **-9.2** | -6.977 | 6.309 | 5.957 |
| 14 | Aranciamycin | 15177995 | **-9.2** | -7.41 | 4.932 | 5.943 |
| 15 | Naringenin | 932 | **-9.2** | -8.428 | 6.086 | 5.963 |
| 16 | CDA3a | 139588787 | **-9.3** | **-15.723** | **7.273** | **6.661** |
| 17 | Carquinostatin A | 10065662 | **-9.3** | -9.138 | 5.915 | 5.983 |
| 18 | Q27160026 | 115005 | **-9.3** | -8.021 | 6.189 | 5.981 |
| 19 | Diazaquinomycin A | 122105 | **-9.3** | -7.937 | 6.914 | 5.968 |
| 20 | Diazepinomicin | 9868980 | **-9.3** | -9.533 | 7.057 | 5.961 |
| 21 | Yanuthone D | 10436112 | **-9.4** | -9.945 | 6.547 | 5.998 |
| 22 | Anthrabenzoxocinone | 9868865 | **-9.5** | -7.777 | 6.195 | 5.956 |
| 23 | CHEBI:156389 | 123132247 | **-9.5** | -7.965 | 5.502 | 5.961 |
| 24 | Benastatin A | 126408 | **-9.5** | -9.986 | 6.627 | 5.956 |
| 25 | Enterobactin | 34231 | **-9.5** | -8.592 | 5.616 | 5.979 |
| 26 | Parabactin | 126461 | **-9.6** | **-12.559** | 6.59 | **7.095** |
| 27 | Dihydromaltophilin | 101934630 | **-9.7** | -8.295 | 5.809 | 5.969 |
| 28 | Isorenieratene | 9984420 | **-9.7** | -9.533 | 6.071 | **6.059** |
| 29 | A33853 | 133379 | **-9.8** | -8.787 | 6.734 | 5.976 |
| 30 | Rebeccamycin | 73110 | **-9.8** | -10.05 | **7.284** | 6.017 |
| 31 | Cryptophycin 327 | 11422411 | **-10.1** | -10.351 | 6.637 | **6.132** |
| 32 | Porphyrinione | 6438546 | **-10.1** | --- | **7.373** | **6.283** |
| 33 | Ergovaline | 104843 | **-10.2** | -8.029 | 6.56 | 6.009 |
| 34 | Griseusin A | 16102131 | **-10.4** | -7.802 | 5.327 | 5.963 |
| 35 | Oviedomycin | 5323531 | **-10.4** | -7.324 | 5.634 | 5.958 |
| 36 | Melanin | 6325610 | **-10.4** | -7.909 | 6.912 | 5.958 |
| 37 | Erdasporine A | 102584105 | **-10.6** | -9.121 | 7.034 | 5.971 |
| 38 | Chartreusin | 5281394 | **-11.3** | -9.303 | 6.813 | **6.133** |
| 39 | Esmeraldin B | 443757 | **-12.3** | **-11.846** | **7.288** | **6.028** |
| 40 | Rhodomycin A | 9896436 | **-15.4** | -10.473 | 5.345 | **6.947** |
| 41 | Linearmycin A | 15238099 | **-55.7** | -9.501 | 4.116 | **6.548** |

*Bold text indicates compounds that are better than the reference compound

**Table 2**
**ADMET analysis of the reference and screened lead compounds.**

| S.N. | Compound Name | Lipinski's Rule of Five (Ro5) | | | | ADMET | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MW | nHA | nHD | logP | HIA | F20% | F30% | PPB | BBB | CL | Carcinogencity | hERG blocker | Rat Oral Toxicity |
| 1 | Vandetanib | 474.110 | 6 | 1 | 4.852 | --- | --- | --- | 94.049 | ++ | 5.098 | - | +++ | - |
| 2 | CHEBI:156389 | 387.130 | 8 | 4 | 2.654 | - | --- | +++ | 72.315 | -- | 2.494 | - | --- | -- |
| 3 | Oviedomycin | 350.040 | 7 | 3 | 1.735 | +++ | --- | +++ | 86.55 | --- | 0.582 | --- | --- | --- |
| 4 | Esmeraldin B | 652.200 | 11 | 4 | 6.675 | --- | --- | -- | 101.25 | --- | 0.489 | + | -- | -- |
| 5 | Diazaquinomycin A | 318.100 | 5 | 0 | 2.385 | --- | --- | --- | 88.424 | + | 7.64 | + | --- | -- |
| 6 | Aranciamycin | 544.160 | 12 | 5 | 1.901 | +++ | --- | +++ | 87.99 | --- | 1.253 | - | --- | -- |
| 7 | Anthrabenzoxocinone | 460.150 | 7 | 4 | 5.160 | --- | +++ | + | 100.07 | --- | 5.338 | -- | -- | -- |

MW (Molecular Weight): Optimal range 100-600, nHA (Number of hydrogen bond acceptors): Optimal range 0-12, nHD (Number of hydrogen bond donors): Optimal range 0-7, logP (Logarithm of the n-octanol/water distribution coefficient): Optimal range 0-3 log mol/L, HIA (Human intestinal absorption), F20% or F30% (Human oral bioavailability of 20% and 30%, respectively), PPB (Plasma protein binding): ≤ 90%: excellent; otherwise: poor, BBB (Blood-brain barrier), Clearance: ≥ 5: excellent; < 5: poor. The prediction probability values are transformed into six symbols: 0-0.1(---) excellent, 0.1-0.3(--), 0.3-0.5(-), 0.5-0.7(+) medium, 0.7-0.9(++) and 0.9-1.0(+++) poor.

**Machine Learning Analysis:** ML-SF such as KDeep, GNINA and RF, aids drug discovery by predicting molecular properties and protein-ligand interactions, enhancing virtual screening and binding affinity prediction[27]. Table 1 presents the revalidation scores for vandetanib and lead compounds using these ML-SFs. KDeep evaluates protein-ligand interaction based on binding affinity, where a more negative ΔG value indicates stronger binding. Among the 41 compounds analysed, Curamycin A, CDA3a, Parabactin and Esmeraldin B exhibited more negative dG values than vandetanib (-11.386 kcal/mol). GNINA considers the convolutional neural network (CNN) affinity, with higher scores suggesting stronger predicted binding interactions.

CDA3a, Rebeccamycin, Porphyrinione and Esmeraldin B demonstrated better affinities compared to vandetanib (7.202 kcal/mol) in GNINA analysis. RF scoring prioritizes compounds based on predicted bioactivity, with higher pKa values indicating better performance. In this analysis, Urdamycin E, Splenocin C, Curamycin A, Neoantimycin, CDA3a, Parabactin, Isorenieratene, Cryptophycin 327, Porphyrinione, Chartreusin, Esmeraldin B, Rhodomycin A and Linearmycin A had higher RF scores than vandetanib (6.025). It is worth mentioning that the results indicate that CDA3a and Esmeraldin B outperformed vandetanib across all scoring functions.

**ADMET Property Analysis:** The drug discovery and development process are inherently complex, involving significant costs, target identification and extensive pre-clinical and clinical trials[8]. Thus, ensuring the efficacy and safety of a drug to manifest its therapeutic effects in the body is crucial[33]. Parameters like Lipinski's Rule of Five (Ro5), molecular weight (MW), hydrogen bond donors (nHD) and acceptors (nHA) and logP (lipophilicity) are pivotal for assessing drug-likeness.

Additionally, factors including human intestinal absorption (HIA), fractions absorbed (F20% and F30%), plasma protein binding (PPB), blood-brain barrier penetration (BBB), clearance (CL), carcinogenicity, hERG blocking potential (cardiac safety) and rat oral toxicity were evaluated. Only compounds meeting stringent criteria for these parameters, detailed in table 2, are considered suitable for advancing in drug development, ensuring both efficacy and safety in therapeutic applications.

**Interaction analysis and anti-cancer activity prediction:** PLIP interactions of reference and lead compounds, assessed through ADMET analysis, are summarized in table 3. According to the PLIP interaction profile, vandetanib, the reference compound, exhibited fewer interactions such as four hydrophobic, three hydrogen bonds and one cationic interaction with RET protein as in fig. 1a. In contrast, esmeraldin B showed highly favourable interactions with the RET protein, involving seven hydrogen bonds, sevem hydrophobic interactions and two salt bridges (Figure 1b). This highlights a clear relationship between increased hydrogen and hydrophobic interactions in protein-ligand complexes of the hit compound facilitating enhanced binding affinity[34].

Collectively, esmeraldin B stands out with the highest interaction count, superior docking score, validation across all three ML-SFs and satisfying ADMET properties positioning it as a prime candidate. Further, the anti-cancer potential of the compound was evaluated using the PASS prediction algorithm, as detailed in table 4.

The results indicate a higher probability of active outcomes compared to inactive ones, suggesting a strong likelihood of experimental activity for these compounds[10]. The PASS predictions align with findings of significant antineoplastic activity in lung cancer, confirming the potential therapeutic efficacy of the evaluated compounds.

**Scaffold analysis:** The structural representation of both vandetanib and Esmeraldin B is depicted in figure 2. Literature evidence suggests that vandetanib's quinazoline scaffold targets kinases in tumor growth and angiogenesis, enhancing its overall binding potency[17] (Fig. 2a). Esmeraldin B, a phenazine metabolite from Streptomyces antibioticusTü 2706, features a core structure with three aromatic rings and a 5,10-diaza-anthracene arrangement, known for its potent antitumor properties (Fig. 2b).

This scaffold that showcases characteristic of phenazine derivatives, exhibits diverse biological activities including antibacterial effects and significant antitumor activity attributed to its redox properties[29].

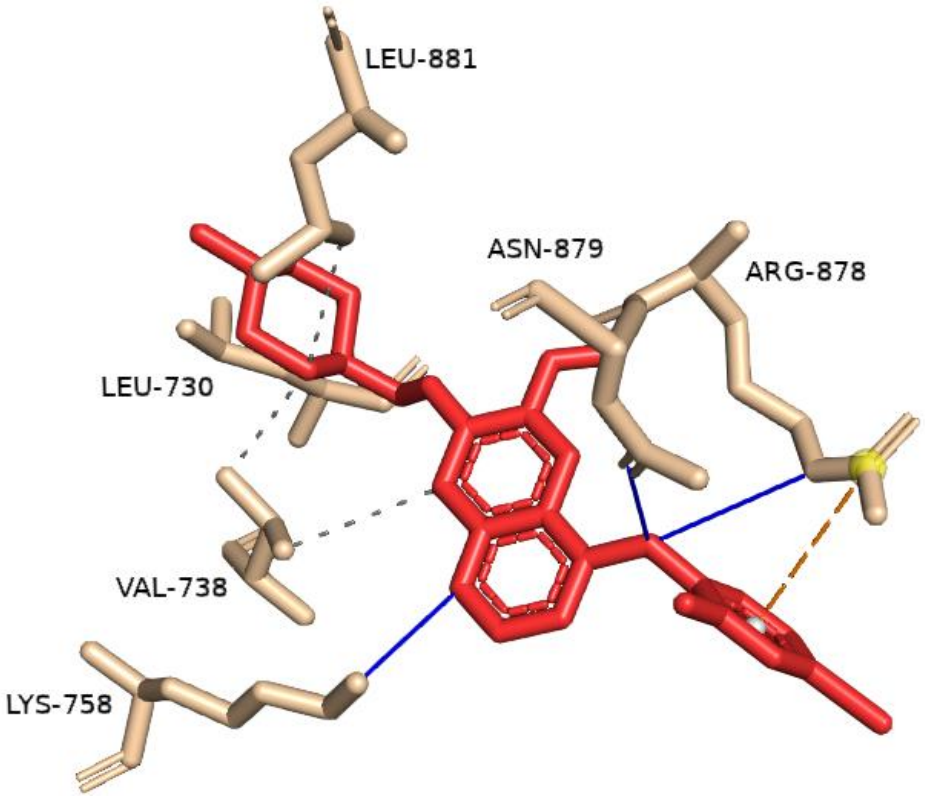
Our study highlights Esmeraldin B's efficacy in inhibiting tumor growth, particularly in combination with other anticancer drugs, aligning with the findings from experimental tumor model studies. Studies have revealed that phenazine-5,10-dioxide derivatives, akin to Esmeraldin B, enhance antiproliferative effects in combination with cisplatin by inducing DNA damage and inhibiting DNA topoisomerase II, making them promising candidates for targeted cancer therapies[35].

**Table 3**
**PLIP analysis detailing interactions of reference and hit compounds.**

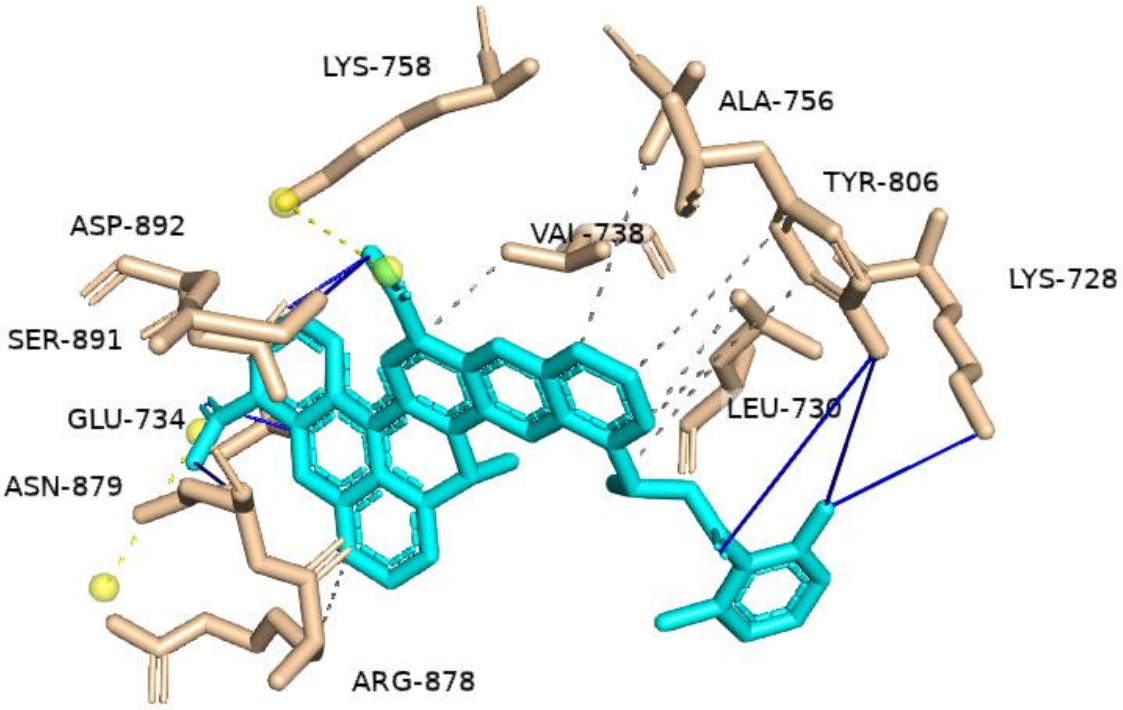
| S.N. | Compound name | Compound ID | No. of hydrophobic interactions | No. of hydrogen bonds | No. of salt bridges |
|---|---|---|---|---|---|
| 1 | Vandetanib | 3081361 | 4 | 3 | 0 |
| 2 | CHEBI:156389 | 123132247 | 8 | 5 | 1 |
| 3 | Oviedomycin | 5323531 | 6 | 4 | 0 |
| 4 | Esmeraldin B | 443757 | 7 | 7 | 2 |
| 5 | Diazaquinomycin A | 122105 | 7 | 3 | 0 |
| 6 | Aranciamycin | 15177995 | 4 | 5 | 0 |
| 7 | Anthrabenzoxinone | 9868865 | 5 | 5 | 0 |

**Table 4**
**Antineoplastic activity of the compounds analyzed using PASS server.**

| S.N. | Compounds | Pa | Pi | Predicted activity |
|---|---|---|---|---|
| 1 | Vandetanib | 0.177 | 0.111 | Antineoplastic (non-small cell lung cancer) |
| 2 | Esmeraldin B | 0.170 | 0.085 | Antineoplastic (lung cancer) |

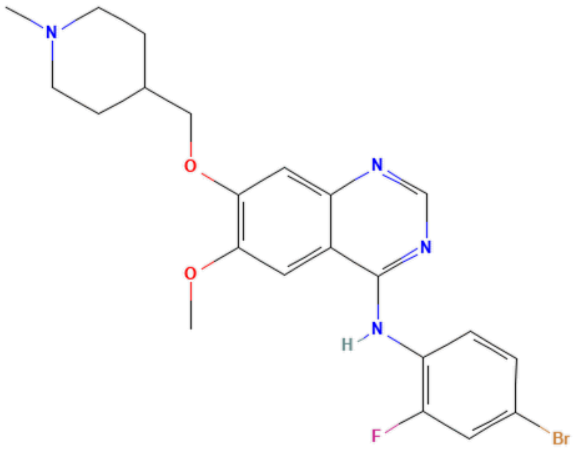



**(a)**

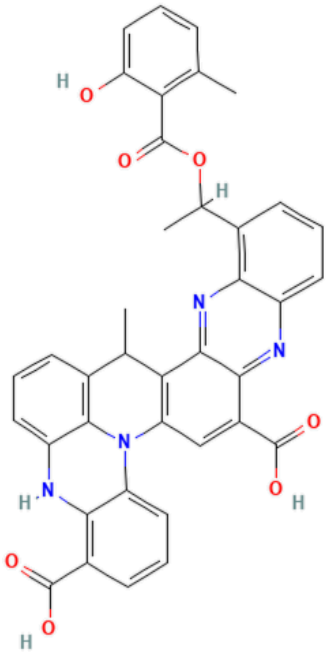



**(b)**

**Figure 1: Interaction profile of RET protein (Sandal) in complex with (a) Vandetanib (Red) and (b) Esmeraldin B (Cyan)**



**(a)**



**(b)**

**Figure 2: The 2D structures of (a) Vandetanib and (b) Esmeraldin B**

## Conclusion

The RET gene, often implicated in non-small cell lung cancer (NSCLC), undergoes mutations leading to uncontrolled cell proliferation. Traditional treatments like vandetanib, although targeting RET kinase, show limited efficacy and notable side effects. This study aims to discover alternative RET inhibitors using secondary metabolites from Streptomyces species. Utilizing the antiSMASH database, 3383 metabolites were identified and docked against the RET protein 2IVU. Compounds surpassing vandetanib's docking score were further analyzed for ADMET properties and validated through machine learning tools like KDeep, GNINA and RF score analysis. Esmeraldin B, a phenazine metabolite, emerged as a promising candidate, demonstrating superior binding affinity and interaction with the RET protein.

Structural analysis revealed Esmeraldin B forming more hydrogen and hydrophobic bonds compared to vandetanib, potentially enhancing its efficacy. The phenazine structure of Esmeraldin B, known for its antitumor properties, further supports its potential as a RET inhibitor. Its significant antineoplastic activity, coupled with favourable ADMET properties, underscores its promise in treating RET-induced NSCLC. This investigation not only highlights the potential of microbial metabolites in cancer therapy but also highlights the importance of integrating computational and biochemical methods in drug discovery. Further *in vivo*

studies and clinical trials are essential to validate Esmeraldin B's therapeutic efficacy and safety, paving the way for novel treatments in lung cancer.

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