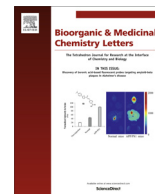




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Identification of inhibitors for vascular endothelial growth factor receptor by using dynamic combinatorial chemistry

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ABSTRACT

The novel analysis method consisting of size-exclusion chromatography (SEC) and HRMS analysis was firstly applied in the discovery of potential inhibitors towards cancer drug targets. With vascular endothelial growth factor receptor (VEGFR-2) as a target, dynamic combinatorial libraries (DCLs) were prepared by reacting aldehydes with amines. Four sensitive binders targeted VEGFR-2 were directly isolated from the library. Antitumor activity test in vitro and inhibition experiments toward angiogenesis were also carried out.

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Over the past decade, a great deal of efforts have been made to generate efficient lead compounds. Generally, the preferred methods used for this strategy are high-throughput screening,¹ rational design,² and fragment-based approaches.³ Due to its higher efficiency, the latter has been increasingly a powerful strategy to identify ligands for biological targets.^{4,5} Meanwhile, a novel fragment-based methodology, dynamic combinatorial chemistry (DCC) was developed. Typically, analog compounds in traditional approaches need to be synthesized individually and subsequently evaluated in a parallel fashion. However, DCC allows the chemical synthesis with biological tests in one single pot. Prior to addition of external templates, the concentration of library members is dictated by its relative stability.^{6–8} Upon addition of a target, the equilibrating species interact with the template via non-covalent interactions, thereby leading to selection and amplification of the strongest binders in the DCLs at the expense of other species.⁹ Recent advances include the discovery of aspartic protease endothiapepsin inhibitors,¹⁰ diselenide exchange reaction,¹¹ and the study of dynamic boronic acid/boronate ester systems.¹² Consequently, DCC has considerable potential as a tool for the discovery of new ligands targeted bioactive molecules.

Larger DCLs are necessary for screening high bioactive compounds. However, a particular important challenge in DCC is the associated analytical methods. Although high-resolution

techniques, such as NMR spectroscopy and X-ray crystallography are applied in the ligand identification, library size is limited to tens of compounds at most due to restricted analytic ability.^{10,13,14} Our research group has used HRMS analysis coupled with size-exclusion chromatography (SEC) to identify the inhibitors of BSA and lysozyme.^{15,16} New highly sensitive binders were directly isolated from the related DCLs, which demonstrated that SEC–HRMS method has emerged as a powerful tool to complement the lead identification in the drug discovery field. However, this method has not been used in the screening of inhibitors aimed at drug targets.

Protein kinase plays a vital role in the regulation of different cellular processes mainly through catalyzing endocellular phosphorylation reaction of specific amino acids, which are closely related to the growth of tumor. Recently, inhibitors of receptor protein tyrosine kinases (RPTKs) have drawn much attention due to its importance in the treatment of cancer.¹⁷ Therefore, it is very significant to explore effective and targeted inhibitors of protein kinase. VEGFR-2 has emerged as an important receptor protein tyrosine kinase in the past several years, thus considerable efforts have been put into developing efficient inhibitors targeted at VEGFR-2.¹⁸ In the previous research, studies concerning 6-urea-3-substituted vinyl indazole compounds have been intensively conducted. Moreover, Schiff base group in compound **1** was replaced by ethylene groups, resulting in compound **2**. Interestingly, it was profitable to exert an anti-neoplastic effect when the R¹ and R² (Fig. 1) groups were occupied by aryl groups.¹⁹ However, tedious

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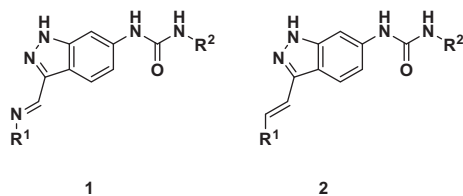


Figure 1. General structural formula of 6-urea-3-substituted vinyl indazole.

and immense work is necessary if a traditional drug screening process is employed. Besides, the synthesis and indeed biological screening of bioactive ligands are very expensive, in term of time, money and resources.

In this study, we reported our recent progress in identifying the most potential compounds through the establishment of DCLs, combining with the SEC–HRMS analysis method.

Initially, DCL 1 (Scheme 1) was designed based on analogous derivatives by reacting aldehydes A1–A6 with amine B1–B17. Some aliphatic amines and cyclamines were selected. Besides, isomers of these aliphatic amines were also added in the library. Occasionally, two heterocyclic compounds were included in the library. Interestingly, 365.0921 was observed in the HRMS results (Fig. 2), while, no ligand was detected in the control test. These results demonstrated that A3B1 or A3B2 might be the potential inhibitors. On the contrary, imines formed between aliphatic amine and aldehydes were not identified from the library.

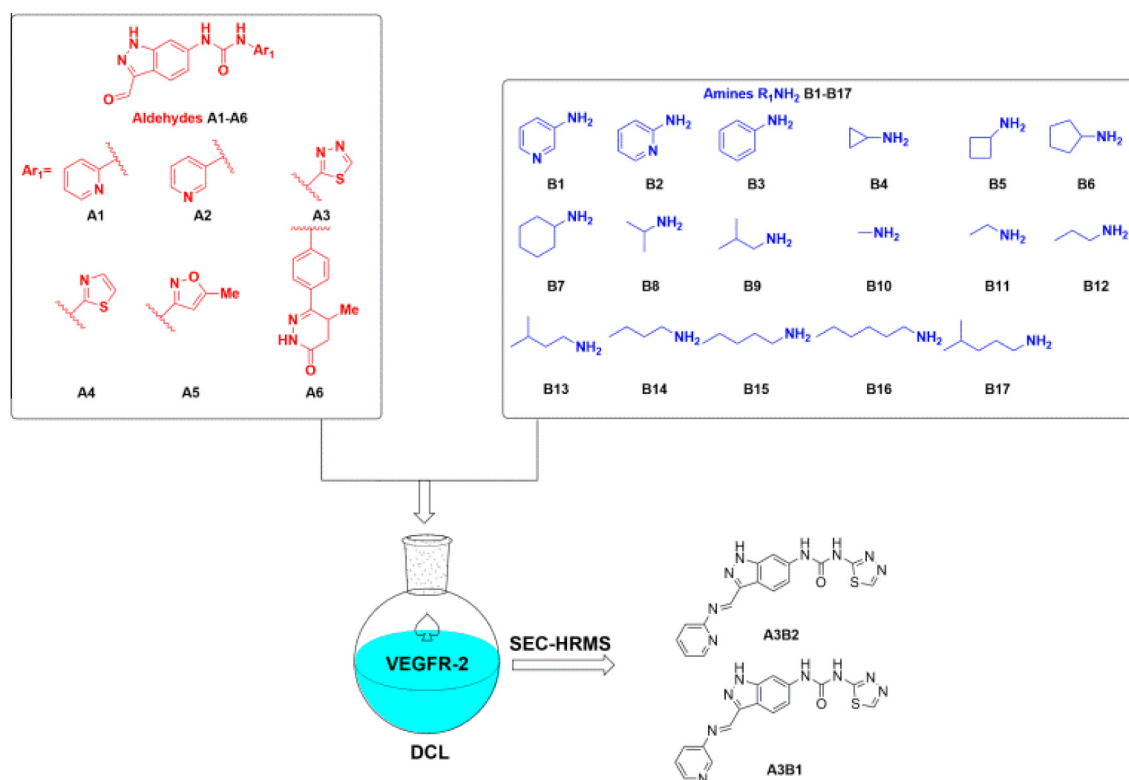
In theory, the larger the library designed, the higher the probability of discovering potential binders towards targets. Thus, a larger DCL was prepared by adding more aldehydes and amines into the library. Five aromatic aldehydes were supplied. However, the formed imines showed worse binding ability towards VEGFR-2 when R¹ was occupied by aryl groups. In addition, more heterocyclic compounds were also added in the DCL 2 (Scheme 2) allowing for the interesting results revealed in the previous section. A

blank experiment proceeded with the same protocol without VEGFR-2 as a target. Interestingly, no molecular ion peaks were detected in the HRMS results. On the contrary, the same molecular ion peak (365.092) was detected again in the HRMS analysis of DCL 2 with VEGFR-2 as a target (Fig. 3). Moreover, another molecular ion peak (473.1501) was observed. Through the analysis of library components, four compounds with the corresponding molecular weight detected in the HRMS were discovered. Two other potential binders (A6B27 and A4B21) were observed apart from A3B1 and A3B2. Therefore, further investigation was necessary for identifying the real binders in the library.

To further validate the bioactivity of these selected compounds, inhibiting effect of four potential binders in vitro was determined according to classic MTT method.²⁰ T lymphocyte leukemia cells (Hun 78), human lung cancer cells (A546), human prostate cells (PC3), human breast cancer cells (MDA-MB-435), human colon cancer cells (HT-29), human gastric cancer cells (BGC-823) were applied in the biological activity assay. IC₅₀ values were calculated according to BLLIS method.²¹

As revealed by the data in Table 1, A3B2 showed better inhibitory activity towards MDA-MB-435 and HT-29, while, A3B1 showed better inhibitory activity towards A546 and PC3. Similarly, a discriminating inhibitory activity was observed when A6B27 and A4B21 were added into the culture medium. Generally, different growth and metabolic mechanism in various cells resulted in diverse therapeutic effect when one anti-cancer drug was applied in the treatment of various cancers. Despite all this, the suppression tests still displayed that the screened compounds showed a moderate to good inhibitory activity towards common cancer cells compared with Sunitinib.

Another important feature of inhibitors targeted VEGFR-2 was inhibition rate toward neoangiogenesis. Thus, inhibitory experiments towards chick chorioallantoic membrane angiogenesis model were also conducted in vitro. When the chicken embryo was incubated for 1 week, chick chorioallantoic membrane was



Scheme 1. Synthesis of DCL 1 targeted VEGFR-2.

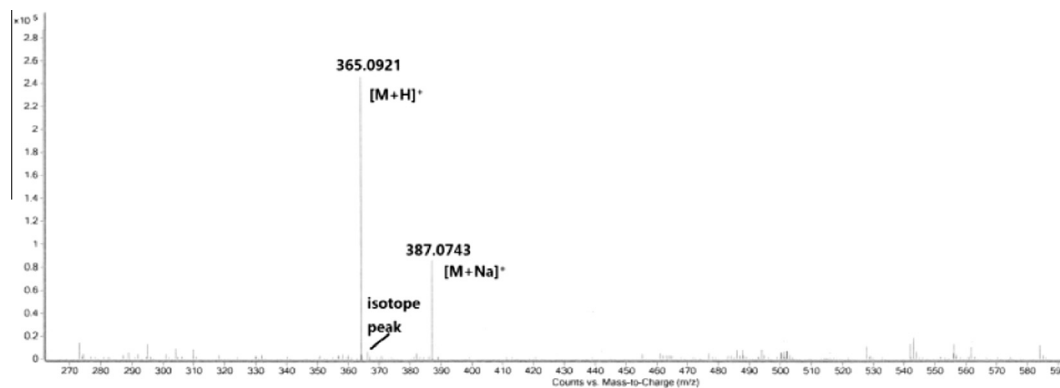
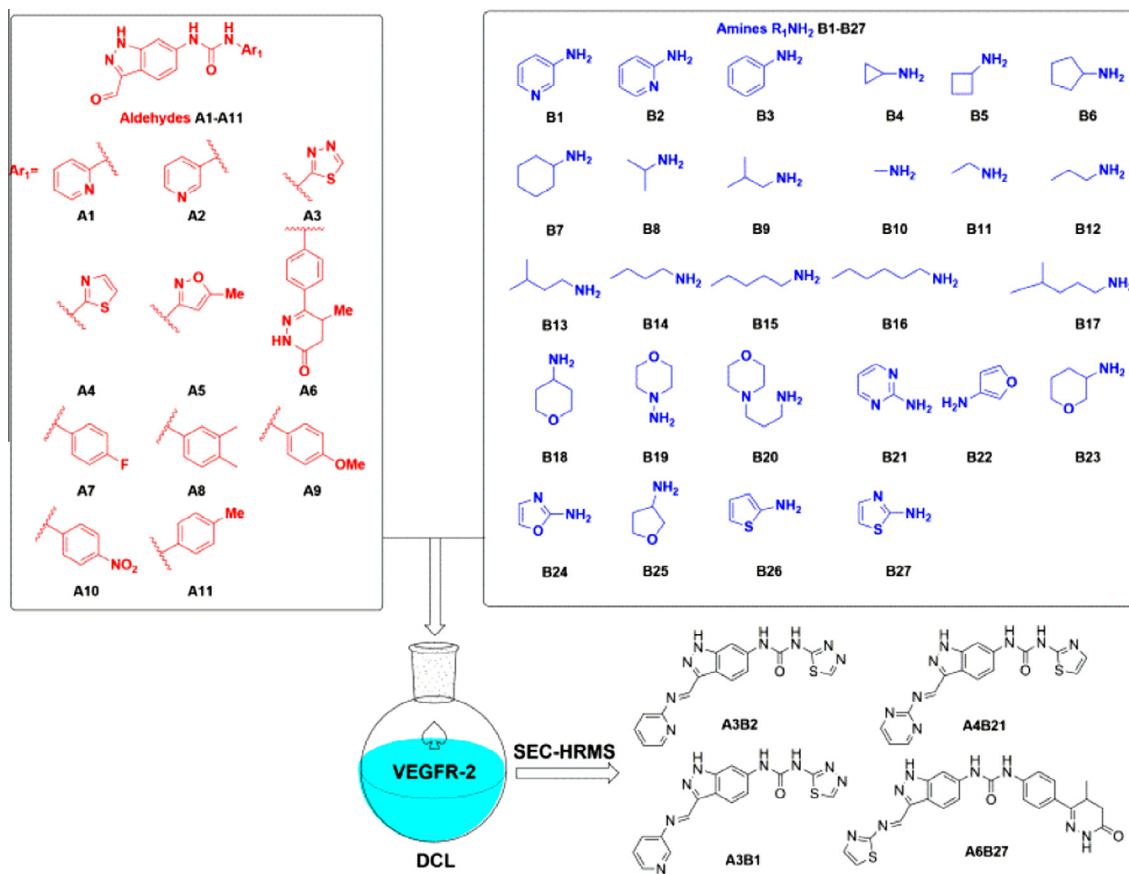


Figure 2. HRMS results of DCL 1 after denaturing of isolated adducts.



Scheme 2. Synthesis of DCL 2 targeting binding to VEGFR-2.

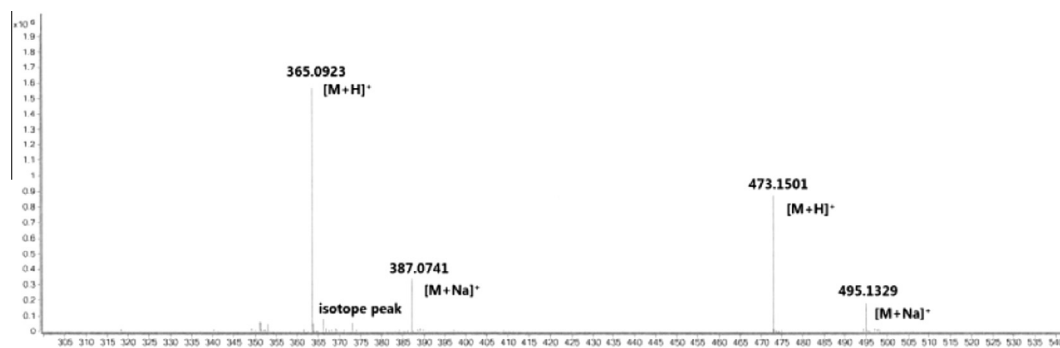


Figure 3. HRMS results of DCL 2 after denaturing of isolated adducts.

Table 1
Antitumor activity data (IC₅₀/μmol L⁻¹) of potential binders in vitro

	Hun 78	A549	PC3	MDA-MB-435	HT-29	BGC-823
A3B2	>100	56.4	>100	4.8	9.4	>100
A3B1	>100	2.4	7.8	55.6	12.1	67.2
A6B27	11.2	8.9	5.6	22.7	12.8	78
A4B21	15.4	19.6	6.8	55.2	78.6	12.8
Sunitinib	—	9.1	55.6	9.3	1.7	—

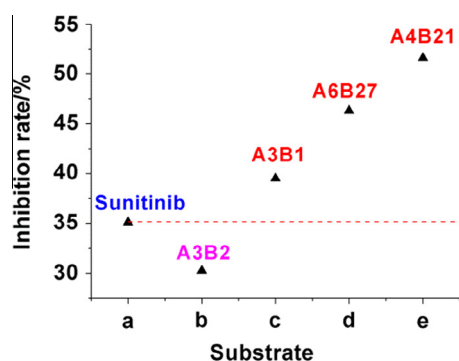


Figure 4. Inhibition rate of potential binders towards angiogenesis.

exposed based on standard operation. Sterile methyl cellulose filter paper plate was placed in the testing region. After incubation at 37 °C for 3 days, vascular branch number was calculated. Sunitinib was selected as a control group. Interestingly, a difference between A3B1 and A3B2 was observed (Fig. 4), which suggested that different substitution positions in the pyridine ring showed different inhibitory effect. A3B2 showed an inhibition rate lower than Sunitinib, while, A3B1 displayed an inhibition rate higher than Sunitinib. Meanwhile, highest inhibition rate (51.60%) was acquired when A4B21 was applied in the inhibitory experiments.

In conclusion, four new sensitive binders targeted VEGFR-2 were directly screened through SEC–HRMS method. A DCL of 102 components was generated under thermodynamic control. Potential binders from DCL 1 suggested that Schiff bases formed between aldehydes and heterocyclic amines might be more sensitive to VEGFR-2. Thus, a larger library was prepared by adding more heterocyclic amines into the library. Two other new potential binders were observed from DCL 2. Antitumor activity test in vitro and inhibition experiments toward neoangiogenesis were then performed. Results revealed that the four components showed a moderate to good inhibitory activity towards common cancer cells. In addition, A3B1, A6B27 and A4B21 displayed a higher inhibition rate towards chick chorioallantoic membrane angiogenesis model compared with Sunitinib. This novel methodology offers great potential in lead compounds discovery against protein targets. Meanwhile, the one-pot mini-library thermodynamically driven approach to identify substances binding VEGFR-2 will be favorable for complementing early-stage discovery in contemporary medicine chemistry program.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2016.02.063>.

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