AncPhore: A versatile tool for anchor pharmacophore steered drug discovery with applications in discovery of new inhibitors targeting metallo-β-lactamases and indoleamine/tryptophan 2,3-dioxygenases

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Abstract We herein describe AncPhore, a versatile tool for drug discovery, which is characterized by pharmacophore feature analysis and anchor pharmacophore (i.e., most important pharmacophore features) steered molecular fitting and virtual screening. Comparative analyses of numerous protein–ligand complexes using AncPhore revealed that anchor pharmacophore features are biologically important, commonly associated with protein conservative characteristics, and have significant contributions to

Abbreviations: AMPC, asian mouse phenotyping consortium; AP, anchor pharmacophore; AR, aromatic ring; AUC, area under the curve; BACE1, beta-secretase 1; BRD4, bromodomain-containing protein 4; CA, carbonic anhydrase; CA2, carbonic anhydrase 2; CatK, cathepsin K; CDK2, cyclin-dependent kinase 2; CTS, cathepsins; CV, covalent bonding; EF, enrichment factor; EX, exclusion volume; GA, genetic algorithm; HA, hydrogen-bond acceptor; HD, hydrogen-bond donor; HIV-P, human immunodeficiency virus protease; HIV1-P, human immunodeficiency virus type 1 protease; HY, hydrophobic; IDO1, indoleamine 2,3-dioxygenase 1; IMP, imipenemase; LE, ligand efficiency; MAPK14, mitogen-activated protein kinase 14; MB, metal coordination; MBL, metallo-β-lactamase; MIC, minimum inhibitory concentration; MMP, matrix metalloproteinase; MMP13, matrix metallopeptidase 13; NDM, new delhi MBL; NE, negatively charged center; NP, without anchor pharmacophore features; PO, positively charged center; RMSD, root mean square deviation; ROC curve, receiver operating characteristic curve; ROCK1, rho-associated protein kinase 1; RT, reverse transcriptase; RTK, receptor tyrosine kinase; SBL, serine beta lactamase; SSEL, secondary structure element length; STK, serine threonine kinase; TDO, tryptophan 2,3-dioxygenase; TDSS, torsion-driving systematic search; TNKS2, tankyrase 2; VEGFR2, vascular endothelial growth factor receptor 2; VIM, verona integron-encoded MBL.

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1. Introduction

Efficient discovery of new hit/lead compounds for a specific target protein still is an important task in innovative drug research & development (R&D). As one of the classic computer-assisted drug design methods, pharmacophore approaches have been widely used within the field of drug discovery, which are mainly based on the concept that specific molecular interactions are observed in molecular recognition of a ligand by a biological target. Typically, a pharmacophore model is defined as an ensemble of chemical interactions (e.g., hydrogen bonds, charges, and hydrophobic contacts) and their spatial arrangement, which enables the rationalization of interaction patterns for a target protein or a set of chemically diverse active ligands, and the subsequent application for virtual screening, de novo design, and lead optimization. Many pharmacophore modelling programs have been established, of which some are free for academic users such as Pharao and Pharmer, and have been successfully used in a variety of drug discovery projects. Yet despite these successes, pharmacophore approaches have not achieved their expected full potential, particularly in facing the actual demand with regard to reduce current expensive overall drug R&D costs.

The effectiveness and efficiency of pharmacophore approaches depend largely on the quality of established pharmacophore model and the understanding of the context and significance of each pharmacophore feature. Usually, a pharmacophore model consists of multiple pharmacophore features to represent the specificity of the target protein to recognize structurally diverse ligands. In practical applications, we noted that a very small proportion of the pharmacophore features have most critical contributions in protein—ligand recognition; we referred such features as ‘anchor pharmacophore features’. For example, most clinically useful kinase inhibitors are found to form 2–3 critical hydrogen bonds (i.e., anchor pharmacophore features) with the active-site hinge region, which is a widely recognized characteristic for kinase inhibitor discovery and development. We recently observed that several clinically relevant metallo-β-lactamases, albeit with large sequence differences, contain a specific conservative active-site region involving the anchor pharmacophore features that specifically recognize the β-lactam antibacterials as well as the potent inhibitors; notably, the compounds have less potent metallo-β-lactamase inhibitory activity if they do not fit well with the anchor pharmacophore features. Although the importance of anchor pharmacophore features has been gradually recognized, comprehensive analysis and proper application of such features has been lacking until now.

Herein, we report a versatile tool for pharmacophore-based drug discovery, termed AncPhore. It involves fine-tuned methods to analyze pharmacophore features for protein—ligand complex, ligand or apo-protein, particularly including the relatively complicated metal-coordination feature that was not well-described previously. By large-scale cross-target analyses of pharmacophore features, we found that anchor pharmacophore features are commonly related with the conservative characteristics within the family of target proteins, have important biological significance (e.g., involving in catalytic nature or binding of natural ligands), and have great contributions to the ligand binding affinity, which offer relatively comprehensive, conceptual understanding of anchor pharmacophore features. More distinctly, a new algorithm and a new scoring function were introduced in AncPhore to achieve anchor pharmacophore steered molecular fitting and virtual screening. Performance evaluations on different types of protein targets using the DUD-E dataset revealed that AncPhore had remarkably improved prediction ability when considering the distinctive contributions and diversity of anchor pharmacophore features. Applications of AncPhore led to the discovery of new structurally diverse inhibitors for two classes of clinically relevant metalloenzymes: metallo-β-lactamases (MBLs) and indoleamine 2,3-dioxygenase 1 (IDO1)/tryptophan 2,3-dioxygenase (TDO), which further demonstrated the power of AncPhore particularly in hit/lead discovery. The AncPhore program is freely available on the website https://ancphore.ddtmlab.org.

2. Results and discussion

2.1. Anchor pharmacophore revealed by cross-target analysis

AncPhore involves ten types of pharmacophore features, including hydrogen bond donor (HD), hydrogen bond acceptor (HA), positively charged center (PO), negatively charged center (NE), metal coordination (MB), halogen bonding (XB), aromatic ring (AR), cation–π interaction (CR), hydrophobic (HY), and covalent bonding (CV) features (as graphically shown in Supporting Information Fig. S1). Uniquely, MB, XB, and CR features that are
Figure 1  Anchor pharmacophore features are conservative in target protein family and great contributions to the binding affinity. (A) Analysis of number of pharmacophore features versus binding affinity of >15,000 complex structures revealed a certain degree of relevance between them. (B) Anchor pharmacophore features are commonly conservative within the family of target proteins, as observed for HIV-P, RTK, STK, SBL, MMP, CTS, MBL, and CA family proteins. (C) Comparison of the binding affinity of the protein–ligand complexes with/without anchor pharmacophore features (AP/NP) indicated the features are of significant importance to achieve the high binding affinity.
not considered in most pharmacophore modelling tools were described in detail (Experimental Section). In particular, the MB feature is defined as to resemble hydrogen bonding interactions, i.e., ligand electron donor corresponding to metal ion acceptor with a proper direction (Fig. S1), which is different from that defined in most pharmacophore modelling programs. The optimal distance and angle for MB in different situations are set according to our analyses of metalloenzyme—ligand complexes. The current version of AncPhore can be used to analyze pharmacophore features for a given protein—ligand complex, a ligand or an apoprotein, as described in the Experimental Section.

By using AncPhore, we carried out pharmacophore feature analyses for more than 17,000 protein—ligand complex structures covering >3600 target entries from PDBbind v2019. As shown in Fig. 1A, most protein—ligand complexes had a range from 3 to 15 pharmacophore features, mainly concentrated in 4–10 features. Globally, the binding affinity increased as the number of pharmacophore features increases only when less than 8 pharmacophore features (Fig. 1A). There was no obvious relevance of the number of individual hydrogen bonding, aromatic ring, and metal coordination features with the binding affinity data, with the exception of ionic interaction features (Supporting Information Fig. S2). By comparison, for single target, we can observe a relatively obvious correlation between the binding affinity and the number of pharmacophore features, as analyzed for the targets with >100 complex structures (Supporting Information Fig. S3), suggesting that the pharmacophore features recognized by AncPhore could at least partly reflect the binding affinity content.

We then attempted to analyze anchor pharmacophore features (i.e., most important pharmacophore features) for the target proteins which have a large number of protein—ligand complex structures, including carbonic anhydrase 2 (CA2), human immunodeficiency virus type 1 protease (HIV-1-P), and cyclin-dependent kinase 2 (CDK2). Through structure superimposition and pharmacophore feature analysis, for CA2, we observed two most frequent pharmacophore features: a metal coordination feature (MB) and a hydrogen-bonding acceptor (HA1), which exist in 89.28% and 84.29% of 401 analyzed complex structures, 1934 Qingqing Dai et al.

The overall results provided relatively comprehensive, conceptual understanding of anchor pharmacophore features, namely, that such features are biologically important, commonly associated with protein conservative characteristics, and have significant contributions to the binding affinity. Anchor pharmacophore features are not only able to reflect the nature of biological target proteins evolved to conservatively recognize their natural substrates but also provide key pointcuts for the discovery of physiologically active compounds and drug molecules. In general, anchor pharmacophore features could be identified by pharmacophore feature analysis of sufficient structural information or by investigation of active-site characteristics, e.g., catalytic nature or binding features of natural substrates. The latter manner is previously undervalued and particularly important for the target proteins whose complex structures are rarely available. Although anchor pharmacophore features are focused on protein—ligand interactions in this study, such features should be not confined to protein—ligand recognition. For example, in protein—protein recognition, the ‘hot-spot’ residues are usually observed, which provide key features for specific protein—protein recognition as well as identification of drug-like small molecules to alter the protein—protein recognition20. Analysis of anchor pharmacophore features in protein—protein recognition is worth expecting. Given the importance of anchor pharmacophore features, appropriate treatment of such features in virtual screening, de novo molecular design, and structural optimization is most likely to improve the efficiency of drug discovery.

2.2. Anchor pharmacophore steered virtual screening

We next examined the potential of AncPhore in virtual screening by consideration of the specific contributions of anchor pharmacophore features. In AncPhore, a sophisticated scoring function called APScore was introduced to evaluate the fitness of queried molecules with a given pharmacophore model; APScore
Figure 2  The activity and mode-of-action of new MBL inhibitors identified by AncPhore. (A) The catalytic mechanism of class B1 di-zinc MBLs. (B) Defined anchor pharmacophore features for B1 MBLs. (C) The inhibitory activity of 4, 6, and 9 with VIM-2, NDM-1, and IMP-1 and their fitting modes with the pharmacophore models. (D) The inhibitory activity for 4, 6, and 9 with VIM-2 at three different concentrations of Zn(II) (0, 1, and 100 μmol/L) revealed their difference in the potential of zinc ion chelation in solution. (E) Crystallographic analysis revealed the binding mode of 4 with VIM-2; the mFo-dFc electron density (OMIT maps) around 4 (blue mesh, contoured to 3σ) and view from a crystal structure of the VIM-2:4 complex (PDB code 7CHV). (F) Superimposition of VIM-2:4 and VIM-1:hydrolyzed meropenem (PDB code 5NSI) revealed the importance of anchor pharmacophore features and their intrinsic connection with the substrate binding nature.
characteristically involves the inherent weights for different types of pharmacophore features and especially resizable weights for anchor pharmacophore features (Experimental Section). More uniquely, AncPhore is developed to achieve anchor pharmacophore guided molecular fitting process and final ranking of screened molecules (Experimental Section).

We first evaluated the performance of AncPhore on eight target proteins (including HIV1-P, VEGFR2, ROCK1, AMPC, MMP13, CatK, VIM-2, and CA2) with or without consideration of anchor pharmacophore features for a single pharmacophore model that was generated from an apo-structure or a complex structure. The test dataset for each target was downloaded or generated from DUD-E [16]. Almost for all the tested targets, the area under curve (AUC) values for a single model (either from an apo-structure or from a complex structure) with consideration of anchor pharmacophore features are higher than that without setting the anchor pharmacophore features (Supporting Information Fig. S6); meanwhile, anchor pharmacophore features were observed to play important roles in enhancing enrichment factors in virtual screening (Supporting Information Table S1). We also observed that AncPhore has better performance with average AUC of 0.80 when using the pharmacophore models from complex structures than that from apo-structures (average AUC = 0.69, Fig. S6), possibly implying that the former can better reflect the targets’ features.

Given the flexibility and dynamics of binding sites particularly for metalloenzymes, we then tested the performance of AncPhore by simultaneously considering the specific contributions and diversity of anchor pharmacophore features (i.e., using multiple pharmacophore models). AncPhore manifested markedly improved prediction ability on the tested target proteins with the AUC values ranging from 0.80 to 0.865 (Fig. S6) and the enrichment factors at 1% ranging from 2.27 to 19.15 (Table S1). Notably, for the metalloenzymes MMP13, VIM-2, and CA2, the AUC values by multiple-model manner are 0.865, 0.858, and 0.863, respectively, which are higher than that by single-model manner (Fig. S6). These results suggest that appropriate use of multiple pharmacophore features, even for metalloenzymes which commonly involve complicated anchor metal coordination features (Fig. 1B), will be able to largely improve performance in virtual screening; this constitutes the distinctive advantage of anchor pharmacophore guided strategy in drug discovery.

2.3. Application cases

We subsequently examined the power of AncPhore in virtual screening for MBLs. MBLs are β-lactamases that use zinc ions to activate a nucleophile water molecule to hydrolyze almost all β-lactam antibiotics (Fig. 2A), including the ‘last-resort’ carbapenems, which represent one class of most attractive targets to combat antibacterial resistance [7,8]. The current research focus is to identify new inhibitor chemotypes for broad-spectrum targeting of MBLs, MBLs are commonly involved in complicated anchor metal coordination features (Fig. 1B), which have been demonstrated as essential features for β-lactam substrate recognition. Due to crystallographically observed multiple coordination modes with the active-site zinc ions, we selected six distinct pharmacophore models with defined anchor pharmacophore features (Supporting Information Fig. S7) to screen commercially available compounds with molecular weights less than 350 in the VITAS-M and enamine library. From the top-ranked hit compounds, compounds 1–10 (Table 1) were chosen for experimental validation, since their chemical scaffolds have not been reported as MBL inhibitors to date. The fitting modes and APScore values of 1–10 with the pharmacophore models are shown in Fig. 2C and Supporting Information Fig. S8.

As shown in Table 1, compounds 1-10 displayed varying degrees of inhibitory activity against the representative MBL enzymes: VIM-2, NDM-1, and IMP-1. Of them, four compounds manifested potent inhibitory activity to one or all of the tested enzymes, including 3, 4, 6, and 9. Compound 3 displayed IC₅₀ values of 38.15, 196.0, and 2.37 μmol/L to VIM-2, NDM-1, and IMP-1, respectively. Compound 4 had more potent inhibition than i-captoprll (Table 1) against VIM-2 and NDM-1 with IC₅₀ values of 0.45 and 66.7, respectively (Fig. 2C); the ligand efficiency (LE) values of 4 to VIM-2, NDM-1, and IMP-1 are 0.59, 0.39, and 0.45, respectively (Table 1). The LE value calculated using Eq. (1):

\[
LE = 1.4 \times \frac{pIC_{50}}{N}
\]

where \(N\) is the number of heavy atoms (i.e., non-hydrogen atoms).

Notably, compound 6 manifested potent broad-spectrum inhibition to all the tested enzymes (Fig. 2C); the IC₅₀ values to VIM-2, NDM-1, and IMP-1 are 0.62 μmol/L (LE = 0.51), 0.67 μmol/L (LE = 0.51), and 3.90 μmol/L (LE = 0.45), respectively (Table 1). Compound 9 showed good inhibitory activity to VIM-2 (IC₅₀ = 6.76 μmol/L) and moderate activity to NDM-1 (IC₅₀ = 53.2 μmol/L) and IMP-1 (IC₅₀ = 57.5 μmol/L, Table 1). We further tested the inhibitory activity for 4, 6, and 9 with VIM-2 at three different concentrations of Zn(II) (0, 1, and 100 μmol/L) to examine the potential of zinc ion chelation in solution. With addition of 100 μmol/L zinc ions, compound 4 showed relatively low inhibitory activity to VIM-2 compared with the low concentrations of zinc ions (Fig. 2D). Unexpectedly, the inhibitory activity of 6 to VIM-2 decreased significantly with increasing concentrations of zinc ions (Fig. 2D), suggesting that 6 has strong ability to chelate with zinc ions in solution and may cause non-specific influence on metal ions containing species.

In contrast, there is no obvious differences between the inhibitory activity of 9 with or without excess zinc ions (Fig. 2D), indicating that 9 is not a strong zinc ion chelator in solution. We then tested whether 4, 6, and 9 have potential to reverse resistance of meropenem, a representative carbapenem antibiotic, in Escherichia coli with expression of VIM-2 MBL (Experimental Section). Compound 4 showed more potent activity than 9 in the cell-based assays, which could reduce MIC values of meropenem to 0.125 μg/mL (32 fold) and 0.25 μg/mL (16 fold) when treatment of 100 μg/mL and 10 μg/mL 4, respectively (Supporting Information Table S2). By comparison, compound 6, albeit with potent enzymatic activity, had no obvious cellular activity (Table S2), probably due to its poor cell permeability or other factors.

We then carried out co-crystallization experiments for VIM-2 in complex with 4, which has a highest LE value (LE = 0.59) to VIM-2 (Table 1) and good cellular activity (Table S2). The VIM-2:4 complex structure (PDB code 7CHV) was solved to 2.17 Å resolution, in which 4 was well-modelled with the clearly defined electron density in the active site (Supporting Information Table S3 and Fig. 2E). In VIM-2:4 structure, we observed that the
carboxyl oxygen and N-3 atom of the imidazole ring of 4 are positioned to chelate with the active site Zn2, and its carboxyl group forms electrostatic interactions with Arg228 on the L10 loop, and its phenyl group makes face-to-edge π–π stacking and hydrophobic interactions with Tyr67 on the L3 loop (Fig. 2E). The crystal structure fully demonstrated that 4 binds to form proposed anchor pharmacophore features with Zn2 and Arg228 (Fig. 2E). Subsequently, we used AncPhore for virtual screening against human IDO/TDO enzymes, which are heme-containing dioxygenases that catalyze the oxidative cleavage of the 2,3-indole position of tryptophan (Trp) to generate N-formyl kynurenine (Fig. 3A). These enzymes are first and rate-limiting enzymes of the kynurenine pathway, and implicated in neurodegenerative diseases and tumoral immune resistance; in particular, IDO1 and TDO have been demonstrated as important targets for cancer treatment.

### Table 1: The inhibitory activity (IC$_{50}$) and ligand efficiency (LE) of compounds 1–10 with clinically relevant VIM-2, NDM-1, and IMP-1.

| Compd. | Chemical structure | IC$_{50}$ (μmol/L) | LE |
|--------|-------------------|-------------------|-----|
|        |                   | VIM-2             | NDM-1 | IMP-1 |
| 1      | ![Chemical Structure](image1) | >1000/0.30 | >1000/0.30 | >1000/0.30 |
| 2      | ![Chemical Structure](image2) | 540.0/0.27 | >1000/0.25 | 347.8/0.28 |
| 3      | ![Chemical Structure](image3) | 38.15/0.31 | 196.0/0.26 | 2.37/0.39 |
| 4      | ![Chemical Structure](image4) | 0.45/0.59 | 66.7/0.39 | 15.2/0.45 |
| 5      | ![Chemical Structure](image5) | 168.7/0.31 | >1000/0.25 | 266.9/0.29 |
| 6      | ![Chemical Structure](image6) | 0.62/0.51 | 0.67/0.51 | 3.90/0.45 |
| 7      | ![Chemical Structure](image7) | 402.2/0.32 | 491.2/0.31 | 49.75/0.40 |
| 8      | ![Chemical Structure](image8) | 857.9/0.29 | >1000/0.28 | 398.4/0.32 |
| 9      | ![Chemical Structure](image9) | 6.76/0.52 | 532/0.33 | 57.5/0.42 |
| 10     | ![Chemical Structure](image10) | 962.3/0.28 | >1000/0.28 | 525.8/0.31 |
| L-cap  | ![Chemical Structure](image11) | 2.47/0.56 | 196.8/0.37 | 2.50/0.56 |

*Compounds 1–10 were confirmed by high-resolution mass spectrometry (Supporting Information) and used without further purification.

*The IC$_{50}$ values (n ≥ 3) were measured as described in Experimental Section, and the IC$_{50}$ curves were given in Fig. 2 and Fig. S8.

*The IC$_{50}$/pIC$_{50}$ values of L-captopril are from Ref. 22 which were obtained under the same assay conditions.
Although a number of IDO1 and TDO inhibitors have been reported and several inhibitors are in preclinical development, discovery of new inhibitor chemotypes is still desirable at present. By comprehensive analysis of all reported complex structures for IDO1/TDO and the catalytic mechanisms of the tryptophan substrate (Fig. 3A), we defined an anchor metal-coordination pharmacophore feature (Fig. 3B) and selected 4 pharmacophore models for virtual screening (Supporting Information Fig. S9). By visual inspection and novelty searching, we selected 10 structurally different hit compounds (Table 2) for biological tests from the top-ranked compounds identified by AncPhore, and their fitting modes with the pharmacophore models are shown in Fig. 3C and Fig. S10.

As shown in Table 2, several compounds showed inhibition to IDO1 and/or TDO, of which 16 and 20 are most potent inhibitors for IDO1 and TDO. 16 displayed IC50 values of 6.63 μmol/L.
(LE = 0.35) and 9.55 μmol/L (LE = 0.33) to IDO1 and TDO, respectively; 20 had activity of 43.9 μmol/L (LE = 0.36) and 7.12 μmol/L (LE = 0.42) against IDO1 and TDO, respectively (Table 2 and Fig. 3C). Since IDO1 and TDO have a characteristic absorption peak at 406 nm (Soret band) that can be as an index of the iron electronic state in heme-containing proteins (Fig. 3D)25,30,31, we tested whether the hit compounds bind to the heme-iron of IDO1/TDO by detecting absorption peak signals. We observed a red shift in Soret band for IDO1 and IDO incubated with 16 for 2 h at room temperature (Fig. 3D), similar as that observed for known inhibitors NLG-919 analogue and INCB-024360 (Supporting Information Fig. S11)30, suggesting that 16 is likely to bind through the mode which involves heme-iron coordination probably via aromatic nitrogen atom, consistent with

Table 2 The IC50 and LE values of hit compounds 11–20 with IDO1/TDO.

| Compd. | Chemical structure | IC50 (μmol/L)/LE | hIDO1 | hTDO |
|--------|-------------------|-----------------|-------|------|
| 11     | ![Chemical Structure](image1.png) | >500/<0.33     | 268/0.36 |
| 12     | ![Chemical Structure](image2.png) | >1000/<0.24    | 237.4/0.27 |
| 13     | ![Chemical Structure](image3.png) | >500/<0.24     | 201.9/0.27 |
| 14     | ![Chemical Structure](image4.png) | >500/<0.24     | >500/<0.24 |
| 15     | ![Chemical Structure](image5.png) | ~300/~0.27     | 262.3/0.28 |
| 16     | ![Chemical Structure](image6.png) | 6.63/0.35      | 9.55/0.33 |
| 17     | ![Chemical Structure](image7.png) | 261.7/0.31     | >500/<0.29 |
| 18     | ![Chemical Structure](image8.png) | >500/<0.29     | ~300/~0.31 |
| 19     | ![Chemical Structure](image9.png) | ~280/0.24      | 204/0.25 |
| 20     | ![Chemical Structure](image10.png) | 43.9/0.36      | 7.12/0.42 |
| NLG-919 analogue | ![Chemical Structure](image11.png) | 0.09/0.47 | 0.15/0.45 |

*Compounds 11–20 were confirmed by high-resolution mass spectrometry (Supporting Information) and used without further purification.

bThe method for measuring IC50 values (n ≥ 3) is described in Experimental Section; IC50 curves are shown in Fig. 3 and Supporting Information Fig. S10.

cThe LE values are calculated as Eq. (1).
the proposed mode by AncPhore (Fig. 3C). Similarly, 19 resulted in a red shift in Soret band when incubated with IDO1, possibly indicating that it binds to the heme-iron (Fig. 3E); 19 did not affect the UV–Vis spectrum of heme-iron in TDO, probably due to its weak TDO inhibition (Fig. 3C and E). In contrast, no red shift is observed for 20 complexed with IDO1 or TDO, possibly suggesting that 20 does not involve heme-iron coordination (Fig. 3F). Besides, 17 was also observed to affect the absorption peak signal of heme-iron in IDO1 (Fig. S11). Overall, among these hit compounds, 16 is the most promising starting point for further structural optimization to develop potential drug candidates against IDO1/TDO mediated diseases.

3. Conclusions

This work provided conceptual understanding of anchor pharmacophore features mainly in protein–ligand recognition and detailed description of AncPhore as an effective, versatile tool for drug discovery. The retrospective studies have demonstrated that consideration of the distinctive contribution and diversity of anchor pharmacophore features enables substantial improvements in the performance of AncPhore in hit/lead discovery for various types of target proteins, including metalloenzymes, which contain relatively complicate metal ion-involving interaction modes that are not well-addressed previously. The followed two case studies, 16 and 20, are the most promising starting point for further structural optimization to develop potential drug candidates targeting these two classes of targets. Although anchor pharmacophore features cannot be able to cover all possible binding modes, e.g., allosteric effects, such previously under-valued features are an important part to improve the capacity of pharmacophore approaches in target-centered drug discovery. Moreover, understanding of the importance and diversity of pharmacophore feature contents is highly recommended, which may be one of the inner driving forces for efficient hit/lead discovery and progressive development of pharmacophore modelling approaches.

4. Experimental

4.1. AncPhore description

AncPhore, written in C/C++ programming language, is provided as a versatile tool for pharmacophore-based drug discovery. The main features of AncPhore include pharmacophore feature analysis, and anchor pharmacophore steered molecular fitting and virtual screening. It has a large degree of flexibility to achieve different functions, such as: i) pharmacophore feature recognition and model generation for a ligand structure, an apo-protein structure or a protein–ligand complex structure; ii) ligand conformation generation by torsion-driving systematic search (TDSS); iii) ligand superimposition and similarity calculation according to their pharmacophore features; iv) ligand fitting with a pharmacophore model; v) high-throughput virtual screening by single-model or multi-model manner. An additional PyMol Plugin is provided for graphic display. Detailed descriptions are as follows.

4.1.1. Definition of pharmacophore features

Ten types of pharmacophore features including HD, HA, PO, NE, MB, XB, AR, CR, HY, and CV were defined (Fig. S1). Each pharmacophore feature was labeled with a two-lettered code as shown in Supporting Information Table S4. The Gaussian volume \( V \) and charge \( Z \) are associated with root atoms. Hydrogen bonds are calculated by four categories according to the number of the root atoms.

\[
V = \int p \exp\left(-\frac{|m-r|^2}{\sigma^2}\right) dr
\]

where \( p \) is a scaling constant, \( m \) and \( \sigma \) represents the position and radius of each pharmacophore point, respectively. The HD, HA, MB, XB, AR, and CR features were defined with a direction and a hydrogen-bond acceptor (HA) in an appropriate distance and angle. Typically, the O/N/S/F/Se atom with at least one lone pair of electrons can be regarded as an HA. Herein hydrogen bonds are detected by four scenarios according to the number of the root atoms of HD and HA, and the corresponding cutoff values of \( \theta_1, \theta_2 \), and \( \alpha \) are set as shown in Fig. 4.
Figure 5  Definition of MB and XB. (A) ‘M’ is the active site metal ion(s) in the protein. ‘L’ refers to the ligand atom (O/N/S/F/Se) that is positioned to coordinate with metal ions, and ‘R’ is the root atom of ‘L’. \( d_{L-M} \) is the distance from ‘L’ to ‘M’. \( \theta \) is the directional bond angle, and the purple arrow represents its direction. Metal coordination bonds are calculated by two categories according to the number of ‘L’ root atoms. (B) ‘X’ is the halogen atom (I/Br/Cl) in ligand, ‘A’ refers to the halogen bond acceptor (O/S/N) that has at least one lone pair, and ‘C’ is the carbon atom. \( d_{X-A} \) is the distance from ‘X’ to ‘A’, \( R_X \) and \( R_A \) are the van der Waals radii of X and A, respectively. \( \theta_1 \) is the angle of the ‘A’ relative to the C–X bond, and \( \theta_2 \) is the angle of the ‘X’ relative to the A–C bond. The brown arrow represents its direction.

between a positively charged center and a negatively charged center is less than 4.5 Å.

Since metalloenzymes are widely found in various biological systems, accounting for 1/3 of all natural enzymes, metal coordination (MB) is also one type of important protein–ligand interactions. As observed in our previous study, metal coordination resembles hydrogen bonding interaction, e.g., ligand electron donor corresponding to metal ion acceptor with an obvious direction. We here defined metal coordination bonds in a similar manner as that for hydrogen bonds, which involves two parameters: \( d_{L-M} \) and \( \theta \) (Fig. 5A). By analyzing metalloenzyme–ligand complexes, we observed that \( d_{L-M} \) and \( \theta \) are commonly associated with the ligand atom types and their root atom number (Supporting Information Tables S5 and S6). Consequently, the values of ideal \( d_{L-M} \) and \( \theta \) ranges are empirically derived from crystallographic structure data, as shown in Supporting Information Table S7.

Halogen bond (XB) between covalently bound halogen atoms (XB donor) and Lewis bases (XB acceptor) has important contributions to protein–ligand interactions. Among the diverse Lewis bases, the atoms (O/S/N) possessing at least one lone pair are defined as XB acceptor in protein structures. For ligand, the heavy halogen atoms (I/Br/Cl) are considered as XB donors. XB is defined in ligand–protein complexes according to the distance \( (d_{X-A}) \) and the angle \( (\theta) \) between XB donors and XB acceptors (Fig. 5B), where \( d_{X-A} \) is longer than 2 Å but less than the sum of the van der Waals radii of XB donor and acceptor, and \( \theta_1 \) and \( \theta_2 \) are in the range of 140°–180° and 80°–140°, respectively.

Aromatic ring (AR) involving interactions are another important type of protein–ligand interactions. As shown in Fig. 6A, AR involved in π−π stacking interactions are represented by the mass of the ring and the normal vector perpendicular to the ring plane, which were defined by calculating the smallest set of smallest rings and rings’ aromaticity based on Hückel’s rule. Two kinds of typical AR interactions, i.e., face-to-face and edge-to-face interactions, are defined according to the centroid distance \( (d_{cen}) \) and the angle between ring planes \( (\theta_1, \theta_2) \) (Fig. 6A). The face-to-face interactions were assigned with the \( d_{cen} \) value of ~4.4 Å and the \( \theta_1 \) value lower than 15°, while for the edge-to-face π−π interactions, \( d_{cen} \) is lower than 5.6 Å and \( \theta_1 \) is in a range of 75°–105°, respectively.

Another aromatic ring involving feature is cation–π interaction, which is emerging as one of the driving forces in molecular recognition, and is comparable to hydrogen bonding and ionic interactions. The cation–π interaction involves a positively charged center (PO) and an aromatic system (AR), which can be defined according to the above-described methods. Notably, metal ions in protein or ligand were also defined as cation centers. CR was finally designated according to the distance \( (d) \) and angle \( (\theta) \) between the aromatic ring and the cation center in protein or ligand (Fig. 6B), where \( d \) is lower than 6.0 Å and \( \theta \) is in a range of 0°–45°.

In order to detect the hydrophobic features (HY) in protein–ligand interactions, we first identify the ligand hydrophobic centers by using lipophilic contribution of ligand atoms. Then, we calculate the ratio of hydrophobic residues surrounding around the hydrophobic centers within 6 Å; if the ratio exceeds an empirical cutoff value of 0.4, the hydrophobic center is defined as a hydrophobic feature along with the center position.

As the increasing number of covalent drugs and drug candidates have been reported in recent years, covalent bonding feature (CV) is considered in this study. Currently, four main types of protein residues have been found to be covalently targeted, including cysteine (−SH), serine/threonine/tyrosine (−OH), lysine (−NH2), and glutamic acid/aspartic acid (−COOH). We thus annotated four modes of covalent-bonding modes, and common reactive groups for ligands are shown in Supporting Information Fig. S13. The covalent bond features are defined according to the distance between protein and ligand reactive atoms without consideration of the bond direction.

4.1.3. Pharmacophore feature analysis for apo-protein structure

Here the binding site is first defined according to the co-crystal ligand or automatically identified by cavity detection. Then, a 3D grid with 0.5 Å grid space is used to cover the whole binding site. The HA, HD, PO, NE, and HY features for binding site of apo-protein are defined based on molecular interaction fields, and followed by k-means clustering analysis to yield optimal pharmacophore features. Similarly, the AR features for each aromatic residue in binding site are analyzed within 3.5–6.1 Å and grouped into a patch which is further analyzed by clustering, and the direction is opposite to the normal of residues’ aromatic ring. The CV feature is generated for specific residues, such as cystine, serine, threonine and lysine, if which is positioned in specific environments to form covalent bonds with small molecules. Uniquely, for MB, 16 canonical metal coordination modes such as tetrahedron, square pyramid and octahedron, were firstly defined and established for 16 types of metal ions (Supporting Information Table S8). Then, each protein metal ion with coordination atoms (on protein) was fitted with corresponding canonical metal coordination modes; the resulted vacant positions were defined as MB features.
Two proteins were considered. The proteins were divided into mean square deviation (RMSD), and sequence identity between including aligned secondary structure element length (SSEL), root atoms larger than 1), and translation during pharmacophore superimposition, 4

4.1.4. Pharmacophore feature analysis for ligand
Similar as that used for protein–ligand complex, the O/N/S atom with at least one hydrogen atom was defined as an HD, and the O/N/S/F/Se atom with at least one pair of lone electrons was defined as MB feature. The charged centers in ligands were defined according to the electronically charged atoms of groups (Fig. S12). For example, the carboxylic acid, phosphoric acid and sulfonic acid groups were defined as negatively charged centers, and the atoms were assigned as positively charged centers if their calculated formal charges are larger than zero. For AR, the center of an aromatic ring in the ligand structure was regarded as the pharmacophore point of aromatic ring with a normal vector perpendicular to the ring plane. The HY feature is defined by lipophilic contributions of ligand atoms. The HY feature (for example, a strong metal-binding group is treated with a higher weight than a weak metal-binding group, see Supporting Information Fig. S14), where \( \epsilon \) represents the angle between two pharmacophore normal vectors, \( \sigma_i \) refers to the basic weight for a chemical group in fitting with one pharmacophore feature (for example, a strong metal-binding group is treated with a higher weight than a weak metal-binding group, see Supporting Information Fig. S14), \( q \) is a unit quaternion describing rotation and translation during pharmacophore superimposition, \( \cos(\theta(q)) \) denotes the angle between two pharmacophore normal vectors, \( \theta_0 \) is set as 0 (the number of root atoms equals 1) or \( \frac{\pi}{2} \) (the number of root atoms larger than 1), \( \varphi(\theta(q)) \) represents the direction difference of matching pharmacophore features, and \( p_0 \) represents initial coordinates of the pharmacophore model Y. The largest \( V_{\text{overlap}} \) corresponds to the best pharmacophore superimposition.

4.1.5. Exclusion volume
The exclusion volume constraints were defined according to binding site surface atoms and mainly used to represent the shape of the binding site.

4.1.6. The protocol for cross-target pharmacophore analysis
To analyze cross-target pharmacophore features, we first classified >17,000 protein–ligand complexes collected from v2019 PDBbind database into different groups by comparing protein structural similarity using the MICAN program; those indexes including aligned secondary structure element length (SSEL), root mean square deviation (RMSD), and sequence identity between two proteins were considered. The proteins were divided into one group if their SSEL \( \geq 100\% \), RMSD \( \leq 2.0 \), and sequence identity \( \geq 30\% \). By using this classification criteria, 201 groups were obtained; of them, 77 target protein groups contain at least three structurally similar proteins. Then, all the protein–ligand complex structures in the same group were superimposed onto one same reference complex with aim to compare them in same coordination, followed by executing pharmacophore analysis for each complex to yield a corresponding pharmacophore model. Finally, all generated pharmacophore models in one group were merged and analyzed to obtain cross-target pharmacophore features.

4.1.7. Molecular fitting
The mapping of ligand to reference pharmacophore model is achieved via pharmacophore superimposition between ligand pharmacophore features and reference pharmacophore features by the Largest-Overlapping-Volume-Winning2 and total least-square algorithms, which are briefly described as follows. First, the pair of matching pharmacophore points between two set features are determined if it meets the following requirement as Eq. (3):

\[
\frac{|d_{ab} - d_{cd}|}{\sigma_{a} + \sigma_{b} + \sigma_{c} + \sigma_{d}} \geq \epsilon
\]

where \( \sigma \) represents the tolerance of each pharmacophore point, \( d_{ab} \) is the distance between pharmacophore points a and b in the first pharmacophore model, \( d_{cd} \) is the distance between points c and d in the second pharmacophore model, and \( \epsilon \) is set as 0.5. Following this requirement, all possible combinations of matching pharmacophore pairs are identified. Next, the total least-square optimization algorithm is used to determine optimal rotational angle and axis for all combinations of matching pharmacophore pairs. The total volume overlap \( V_{\text{overlap}} \) of matching pharmacophore pairs between two models is computed using Eq. (4):

\[
V_{\text{overlap}} = \sum_{j=1}^{N} C_i W_i A_i \lambda_i \varphi(\theta(q)) \exp \left( -\frac{q^T P Y q}{\sigma_{i,\lambda} + \sigma_{i,T}} \right)
\]
4.1.8. Scoring
The similarity value between the query and reference pharmacophore model is calculated using Eq. (5):

\[
\text{APS} = \frac{V_{\text{overlap}}}{V_{\text{ref}}} + k_1 \times \frac{n}{N} - k_2 \times \frac{\sum V^2_{\text{overlapEX}}}{V^2_{\text{refEX}}}
\]

where \(V_{\text{overlap}}\) is the largest overlapping volume between the query and reference model, \(V_{\text{ref}}\) corresponds to the volume of the reference pharmacophore model, \(\sum V^2_{\text{overlapEX}}\) is the sum of square of overlapping volumes with exclusion volumes, \(V^2_{\text{refEX}}\) corresponds to the square of exclusion volumes of the reference model, \(k_1/k_2\) refer to the weighting factor with default values 0.25/5, \(n\) is defined as the number of matching pharmacophore pairs, and \(N\) is the number of the reference pharmacophore features. Since some specific chemical groups can be assigned with different pharmacophore features, as, for example, a carboxyl group can represent a negatively charged center, a hydrogen bond acceptor, or a metal coordination feature, we hence use APS as a scoring function to put particular emphasis on the reference pharmacophore model including anchor pharmacophore features, and to avoid the asymmetry of compared features, particularly in virtual screening, which will have more practicability than Tanimoto coefficient.

4.1.9. Ligand conformation generation
Another important step in AncPhore for virtual screening is to efficiently generate rational and diverse conformers for the database ligands of interest. The torsion-driving systematic search (TDSS) and genetic algorithm (GA) were included in AncPhore for ligand conformation generation. The TDSS method generates efficiently generate rational and diverse conformers for the data-base ligands of interest. The torsion-driving systematic search (TDSS) and genetic algorithm (GA) were included in AncPhore for ligand conformation generation. The TDSS method generates allows diverse low-energy conformers by applying torsion-driving rules and graph symmetry with energy and RMSD cutoff, as described by Oboyle et al.\(^3\). The GA method, mimicking the process of natural evolution, is a common method to find a global optimum solution to difficult optimization problem. It may be able to generate diverse conformers stochastically on the basis of either RMSD diversity or energy throughout gene-based evolution, e.g., mutation, crossover and replication; the computation time of conformer searching partly depends on the selection of initial population and empirical parameters.

4.1.10. Computational time
A total of 100 compounds were randomly selected for testing computational time of AncPhore on a single CPU (Intel(R) Xeon(R) CPU E5-2680, 2.40 GHz). It took about 10 and 9 min for conformation generation using TDSS and GA, respectively, and takes 25 s for these compounds in model fitting.

4.1.11. AncPhore availability
In addition to the AncPhore program, a PyMol plug-in on Linux and Window is provided for users to view the generated pharmacophore model and the computation results. The AncPhore program, PyMol plug-in and their usage instructions are freely available in https://ancphore.ddmlab.org.

4.1.12. The strengths of AncPhore
Compared with commonly used pharmacophore tools including Pharao\(^1\), Pharmere\(^2\), PHASE\(^2\), and Pharmit\(^2\), AncPhore has its own strengths and unique characteristics, mainly including (Supporting Information Table S9): (1) involving MB, XB, CR, and CV features that are not included in most pharmacophore modelling tools; (2) possessing an analysis method of pharmacophore features for apo-protein, which can automatically detect cavities as possible binding sites and select corresponding protein residues for pharmacophore model generation; (3) introducing a new algorithm and a new scoring function to achieve anchor pharmacophore steered molecular fitting and virtual screening; (4) supporting to use five models for molecular fitting and virtual screening, with the aim to consider pharmacophore model difference and specificity; (5) having high degree of integration and portability, e.g., with two kinds of algorithms for conformation generation and convenient anchor feature assignment. Therefore, we believe AncPhore should be a specific and useful tool for drug discovery.

4.2. Biological assays

4.2.1. MBL protein expression and purification
The VIM-2 (residues 27–266), NDM-1 (residues 1–270), and IMP (residues 19–246) proteins were expressed and purified as described previously\(^5\). The VIM-2 (residues 27–266), NDM-1 (residues 1–270), and IMP (residues 19–246) proteins were expressed and purified as described previously\(^5\). The VIM-2 (residues 27–266), NDM-1 (residues 1–270), and IMP (residues 19–246) proteins were expressed and purified as described previously\(^5\).

4.2.2. MBL inhibition assays
The experiments were performed at room temperature using Tecan microplate reader and flat-bottom 96-well black plates. Compounds were dissolved into 100 mmol/L with DMSO. All the MBL enzymes were diluted in the assay buffer: 20 mmol/L Tris-HCl (pH 7.5), 200 mmol/L NaCl. The compounds (with 10 different concentrations in 3-fold dilution) were pe-incubated with MBL enzymes for 10 min. The reactions were initiated by adding of the substrate FC5\(^5\). The hydrolysis of FC5 was monitored by reading the fluorescence at \(\lambda_{ex}\) of 380 nm and \(\lambda_{em}\) of 460 nm. All determinations were tested in triplicate. The IC\(_{50}\) values were calculated using GraphPad Prism software.

MBL enzymes supplemented with 1 or 100 \(\mu\)mol/L ZnSO\(_4\) were pe-incubated with compounds for 10 min to examine the effects of zinc ions on compound inhibition. The reactions were initiated by the addition of FC5. The IC\(_{50}\) values were determined as described above.

4.2.3. MIC assays
The pUC7-ISAb125-pelB-VIM-2 plasmid was constructed by multi-step gene cloning and transformed into E. coli DH5\(_{\alpha}\) for susceptibility test. Meropenem was purchased from Dalian Meilun Biotechnology Co., Ltd. Ampicillin was obtained from Sangon Biotechnology Co., Ltd. MHB and MHA were purchased from Solarbio (Beijing, China).

Strains of E. coli DH5\(_{\alpha}\) containing plasmids pUC7-ISAb125-pelB-VIM-2 (E. coli-VIM-2) and ATCC 25922 (as control) were used to assess the ability of inhibitors in restoring the antimicrobial activity of \(\beta\)-lactam antibiotic meropenem. Meropenem were tested alone or in combination with inhibitors at 100 or 10 \(\mu\)g/mL. Minimal inhibitory concentration (MIC) values were determined by the standard broth micro-dilution method according to the Clinical and Laboratory Standards Institute (CLSI, M07-A9, 2012) guideline.

4.2.4. Crystallization and data collection for the VIM-2:4 complex
The VIM-2:4 complexes were crystallized by hanging drop vapor diffusion method as described previously\(^5\). Briefly, 10 mg/mL VIM-2 proteins and 5 mmol/L 4 were incubated in crystallization buffer (20 mmol/L Tris-HCl, pH 7.5, 200 mmol/L NaCl, 0.5 mmol/L tris (2-carboxyethyl) phosphine (TCEP) for 60 min at...
4 °C. One microliter (µL) of protein-inhibitor solution was mixed with 1 µL of precipitant (25%–32% PEG 3350, 0.2 mol/L magnesium formate) and incubated at 20 °C. The crystals were harvested using the cryo-protectant solution 30% (v/v) glycerol, and flash-cooled in liquid nitrogen. The diffraction data were collected at the Shanghai Synchrotron Radiation Facility, and processed using HKL2000. The structure was solved by molecular replacement and refinement using Phenix55 and Coot56 programs. Coordinates and structure factors of the VIM-2-4 complex structure (PDB code 7CHV) have been deposited in the PDB.

4.2.5. IDO1/TDO protein expression and purification
The human IDO1 (residues 12–403) and TDO (residues 19–388) were cloned into pET28a vectors for expression with N-terminally His₆-Tagged proteins. IDO1/TDO were over-expressed in E. coli Transetta (DE3) cells at 37 °C using LB medium supplied with 1 mmol/L 5-AlA and 30 µmol/L hemin chloride in a shaker at 200 rpm. When the OD₆₀₀ value reached 0.8–1.0, the temperature was lowered to 25 °C, followed by addition of 0.5 mmol/L IPTG to the culture to induce protein expression for 6–8 h in a shaker at 150 rpm. Cells were then harvested by centrifugation (20 min, 4000 rpm), resuspended in lysis buffer A (for IDO1: 50 mmol/L potassium phosphate, pH 7.1, 0.3 mol/L sodium chloride, 25 mmol/L imidazole, 5% glycerol; for TDO: 50 mmol/L potassium phosphate, pH 7.8, 0.3 mol/L sodium chloride, 5% glycerol) supplemented with EDTA-free protease inhibitor, and lysed by using an ultrahigh-pressure homogenizer (JNBI). The lysates were clarified by sedimentation (13,000 rpm) for 30 min at 4 °C, and then loaded onto an Ni-NTA column, followed by extensive washing with buffer B (for IDO1: 50 mmol/L potassium phosphate, pH 7.1, 0.3 mol/L sodium chloride, 30 mmol/L imidazole, 5% glycerol; for TDO: 50 mmol/L potassium phosphate, pH 7.8, 0.3 mol/L sodium chloride, 30 mmol/L imidazole, 5% glycerol) to remove nonspecifically binding proteins. The target proteins were eluted with buffer C (for IDO1: 50 mmol/L potassium phosphate, pH 7.1, 0.3 mol/L sodium chloride, 350 mmol/L imidazole, 5% glycerol; for TDO: 50 mmol/L potassium phosphate, pH 7.8, 0.3 mol/L sodium chloride, 250 mmol/L imidazole, 5% glycerol). Fractions containing the purified enzymes were concentrated using Amicon Ultra 10K (Millipore) and then desalted using a HiTrap desalting column (GE Healthcare) into pH 6.5 potassium phosphate buffer (100 µmol/L). The purified proteins were stored at −80 °C before use.

4.2.6. IDO1/TDO inhibition assays
The IDO1/TDO enzymatic activities were tested in the assay buffer: 100 mmol/L potassium phosphate, pH 6.5, 0.01% Triton X-100, 40 mmol/L ascorbic acid, 7 µmol/L methylene blue, and 200 µg/mL catalase. The compounds (10 different concentrations) were pre-incubated with the enzymes for 5 min at 25 °C. The reactions were initiated by addition of L-trp (400 µmol/L) in the assay buffer and incubated 30–60 min at 37 °C. The reactions were stopped by addition of 30 µL 30% TCA and incubated 30 min at 50 °C to convert N-formylkynurenine produced by IDO1/TDO into kynurenine. Then, 90 µL of 2% (v/v) pDMAB in acetic acid were added and incubated for 5 min at room temperature. The absorbance at 480 nm was measured using Tecan microplate reader. All determinations were tested in triplicate.

4.2.7. Optical absorption spectroscopic measurements
Ultraviolet–visible (UV–Vis) absorption spectra of IDO1/TDO in complex with various inhibitors were recorded at room temperature under standard atmosphere using Varianosk LUX multifunctional reader (Thermo Fisher Scientific Co., Ltd.) with a spectral slit width of 1 nm. The samples were prepared with 10 µmol/L ferric IDO1 or TDO and 0.5 or 1.0 mmol/L inhibitors in 100 mmol/L potassium phosphate buffer (pH 6.5). UV–Vis absorption spectra (300–700 nm) were recorded after samples incubated for 2 h at room temperature.

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Author contributions
Qingqing Dai and Yuhang Yan contributed equally. Guo-Bo Li designed this project. Qingqing Dai wrote the programming codes; Qingqing Dai, Gen Li, and Junlin Yu tested the programs. Yuhang Yan, Xiangli Ning, Ji Deng, and Lingling Yang performed the biological tests. Guo-Bo Li and Qingqing Dai wrote the manuscript. All authors have given approval to the final version of the manuscript.

Conflicts of interest
The authors declare no competing financial interest.

Appendix A. Supporting information
Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.01.018.

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