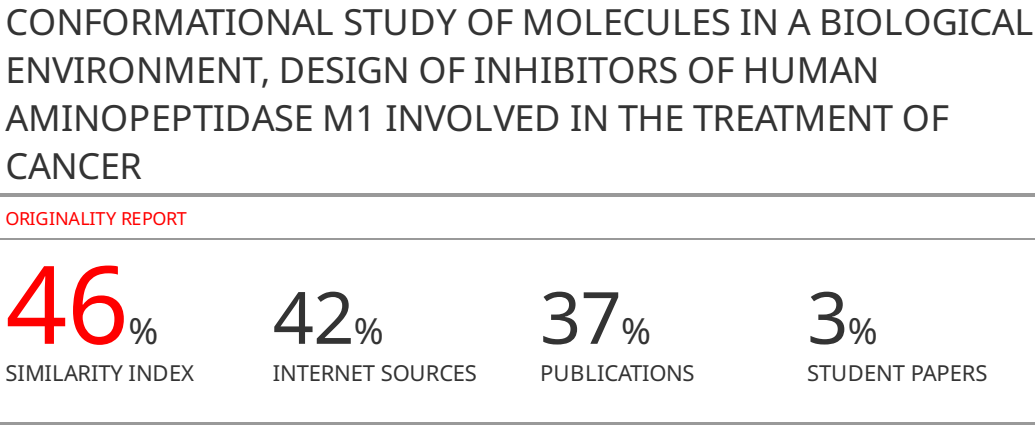
**Reviewer’s Comments**



**CONFORMATIONAL STUDY OF MOLECULES IN A BIOLOGICAL ENVIRONMENT, DESIGN OF INHIBITORS OF HUMAN AMINOPEPTIDASE M1 INVOLVED IN THE TREATMENT OF CANCER**

**ABSTRACT**

**Aim and Objective:** we virtually design novel subnanomolar anticancer hydroxamic acid containing drug candidates, inhibitors of human M1 aminopeptidase (APN) a recent validated target and reaching the predicted subnanomolar range of inhibitory potency.

**Methods:** A quantitative structure activity relationships (QSAR) complexation model has been developed from a series of 37 hydroxamic acid derivatives (AHD1-37 as training set, TS) to establish a linear correlation between the calculated relative Gibbs free energies (GFE: ΔΔGcom) of APN-AHDx complex formation and the experimental inhibition potency (Kiexp). The predictive power of the QSAR model was then validated first with 9 other AHDs not included in the TS and thereafter with the generation of a 3D-QSAR-PH4 pharmacophore (PH4) model to screen the AHD chemical subspace built as a virtual combinatorial library of more than 58,644 AHD analogs). Finally the best PH4 hits were evaluated with the initial QSAR model for predicted potency (Kipre) and pharmacokinetic profile.

**Results:** The QSAR model linear correlation equation: pKiexp = -0.1859×∆∆Gcom + 8.096, R2 = 0.94, the subsequent PH4 model linear correlation between experiment and PH4-estimated IC50: pKiexp =- 1.0006×pKipre – 8.2929, R2 = 0.79 documents the high predictive power of this approach. Finally the screening of the virtual library of AHD analogs yielded 95 orally bioavailable candidates the best reaching a predicted potency (Kipre) of 50 pM and displaying favorable pharmacokinetic profile.

**Conclusion:** The combined use of Molecular Modelling QSAR and in silico PH4-based screening of Virtual Combinatorial Library resulted in novel proposed predicted potent anticancer agent candidates with favorable pharmacokinetic profile.

**keywords**

Drug design, QSAR model, pharmacophore model, complexation model, molecular modeling, ADMET

**INTRODUCTION**

Cancer is one of the most worrying public health concern in the world today. According to GLOBOCAN 2020 studies, more than 19.3 million new cases and 10 million cancer-related deaths were estimated [1]. Although cancer survival rates are expected to improve and cancer mortality rates have declined, cancer remains the first or second leading cause of death in many countries [1]. However, many cancer drugs have undesirable side effects, mainly due to their non-selective activities towards non-cancerous cells [2]. Furthermore, long-term use of anticancer drugs is inevitably accompanied by drug resistance which reduces its effectiveness [3]. Therefore, the development of new anticancer drugs with less toxicity, improved efficacy and higher selectivity continues to be of crucial importance. With growing interest in the discovery of less toxic and more selective anticancer drugs, extensive research has been conducted to identify and characterize cancer therapeutic targets at the molecular level [4].

Aminopeptidase N (APN/CD13) is one of the most studied therapeutic targets associated with cancer [5, 6]. Aminopeptidase N is an enzyme ubiquitous in several human organs, tissues and cell types. It is described as a multifunctional (“moonlighting”) protein with enzymatic functions as well as other functions including antigen presentation and a receptor for certain human viruses (e.g. coronaviruses) [7]. Thus, APN plays an important role in the regulation of protein turnover in almost all organisms [8] [9] [10] [11].

Through multiple studies, a strong correlation between the APN expression level of a cell and its invasive characteristics has been established [12]. Studies have shown that APN dysregulation occurs in almost all types of human malignancies [13]. APN has been observed in several types of cancer, including small cell lung carcinoma [14], thyroid carcinoma [15], acute myeloid leukemia [16], colon carcinoma [17] and prostate carcinoma [18]. APN activity affects metastasis, a complex biological process that contributes to more than 90% of cancer-related deaths, by promoting cell adhesion, cell motility, angiogenesis and extracellular matrix degradation [19] [20].

APN belongs to the zinc-dependent M1 aminopeptidase superfamily of enzymes (MA protease clan) that can be found in all kingdoms of life except viruses [21]. M1 aminopeptidases are characterized by a single zinc ion, a thermolysin fold and two consensus sequence motifs: HEXXHX18E (H = histidine, E = glutamate and X any amino acid) involved in zinc binding and a motif substrate recognition GXMEN [21] [22] [23] [24]. The x-ray crystal structure of human APN was reported by Wong *et al.,* along with the structures of APN related to the generic inhibitors bestatin and amastatin, and an endogenous peptide substrate, angiotensin IV [21]. Human APN is composed of four domains (I-IV) characteristic of the M1 aminopeptidase superfamily [21]. Catalytic domain II contains the zinc-linked consensus motifs 352GXMEN356 and 388HEXXHX18E411 [21]. APN is known as the “black” enzyme [25]. It addresses many peptides, including angio-tensin III and IV, neuropeptides and chemokines [26].

Among the APN inhibitors previously developed as anticancer candidates, bestatin (**1**) is the most studied competitive inhibitor of APN [27] [28]. Bestatin is a natural dipeptide, originally isolated from streptomyces olivoreticuli as an immunomodulatory agent [29]. After clinical trials, bestatin was found to have therapeutic activity against gastric cancer, lung cancer and acute myeloid leukemia, and is currently available in Japan as an adjuvant treatment for acute non-lymphatic leukemia [30] [31] [32] [33]. Another APN inhibitor, Tosedostat (**2**) is an orally bioavailable prodrug that is converted to a pharmacologically active drug inside cells [34]. Tosedostat exhibited an anti-leukemic effect [35]. In previous studies, a series of hydroxamic acid containing compounds were generated, which were inhibitors of *Plasmodium falciparum* M1 aminopeptidase, *Pf*A-M1 [36] [37] [38]. Later in 2019 Jisook Lee *et al.* repurposed the compound (**3**) N-(2-(hydroxyamino)-2-oxo-1-[3',4',5'-trifluoro(1,1'-biphenyl)-4-yl]ethyl)pivalamide reporting it as a novel APN inhibitor more potent than bestatin (**1**) and Tosedostat (**2**). Moreover they synthesized a series of hydroxamic acid inhibitors (**4**) to optimize binding interactions around and beyond the S1' subsite of APN, the most potent being compound (**5**) in the low nanomolar range, Kiexp =  4.5 nM [39].

In the present work, we have built and validated a QSAR ‘complexation’ model based on in vitro activities (Ki) of a training set of 37 selected AHDs against APN human, starting from cristal structure of APN-AHD1 complex (Ki = 4.5 nM, PDB entry 4FYR) [39]. The key ligand-receptor interactions at the active site of APN-AHD1 complex are displayed as a 2D scheme on Figure 2. We have calculated Gibbs free energies of ligand-receptor complex formation (∆∆Gcom) for the TS molecules and correlated them with the observed biological activities. The resulting quantitative structure-activity relationships model (QSAR), which employs the computed parameter ∆∆Gcom was able to explain approximately 94% of the variation in the observed Kiexp. The QSAR model allowed structure-based design of novel AHD analogs. The identified virtual hits reached predicted inhibitory activites Kipre against the APN in the sub nanomolar concentration range. Metrics describing interactions at the active site of APN were assessed from analysis of the X-ray crystal structure of APN (PDB code 4FYR) in complex with one of the most active inhibitors studied in this work (**5**) [39]. The catalytic zinc binding group in the active site is coordinated by a catalytic triad His388, His392 and Glu411 (not shown in the 2D diagram in Figure 2), and the S1 pocket with Asn350, Ala351, Arg363, Gln857 Asp858, Thr860, Ser861, Phe896 and Ser897. Also, a deep hydrophobic pocket S1' with residues Arg381, Ser415, Glu419, Tyr419 Arg442 and Tyr477.

|  |  |
| --- | --- |
| https://lh3.googleusercontent.com/fibzi8JPlQHX2hRxzzYMosO-1GN5sJNqiEth-Rzuqg_Rv3Y0k0Z2GnqgQgnuRDZtyNuWLCqj5DNg_3MNg5Dp4OY4P0-4pFdGLbgTsucsPGL2uwo4qX_6O3WYt8pBsTFj88HS8QuKWHkkKmXs2m7raA | **https://lh4.googleusercontent.com/ZahzutR2fTB5NU5wqwRwtiPEt89N6EETJX0uYjM4AuGVnmJO0GQjzfyVzQDuciYmQ2tJn3WrTbgAQ7f52nsBqA425d34AZEnFrb6CfmZfBKrpHwpvhOaJSeo4Hn2ma00u4F6_yQA2vSlmmn1Sm2JCA** |
| Bestatin (1)  Kiexp =  2370 nM | Tosedostat (2) Kiexp =  1180 nM |
| **https://lh4.googleusercontent.com/LcpJXw4wAv81ugfS5hja8YhP-p0zxi-RzRwbfGJdqpxrp5izAfcDeD39tSdvSqmkq_x2-h-l9-BFgW3diakvqur6HT3FmtC7gWsUkOnYd9u7b3DgFPAMFjHcvPREzH5ap0ulIfxX_fVUfgo8buPtmw** | **https://lh6.googleusercontent.com/5wlJogGVvWG8fnC_pTZjaAnjXbEqJdiSZfp_iTi2ClqGb1y4_bpgzqRPMtNij7kTbYweWcn8SRmY4bqH4xMaweVgBb1MGAhA5YAcKpL7K25WEfH6knPmQL2-DUt4C3ZRm9ExuAncJRAzZkPkYW5tGg** |
| (3)  Kiexp =  118 nM | (4) |
| https://lh5.googleusercontent.com/m_GoYgQm1_wWlWAAxkOa6zmYt3E14WvPcUsB8AxUEzXtPCivw1lonFijIKzHw-I3kEJMTAqKBA-J5eJZzsxkhN7fK4Wqh3jUpNdWH4mEtlwLYWvjUzOqCR95-AfozSQc4ANpiuu1L7GsN7ewUC7X1w |  |
| (5)  Kiexp =  4.5 nM |
| **Figure 1**: Inhibitors of APN. | |

|  |
| --- |
| https://lh6.googleusercontent.com/7M_18YSad36tu5C46Eu_PjPwQCwtq4EsFch5n-KI7Zn4ACDIGbwnFGOg2-0oBZ49oYRUb4PBWEE_48uMQdcuarw2IIYM7_FFU47gQkhStqS3ONBTBfXwPk1-nOXoh4cgkzHclUTqHICDzoidlXHhXg |
| **Figure 2**: *APN*–(4) interactions at active site depicted in 2D |

**METHODS**

**General workflow**

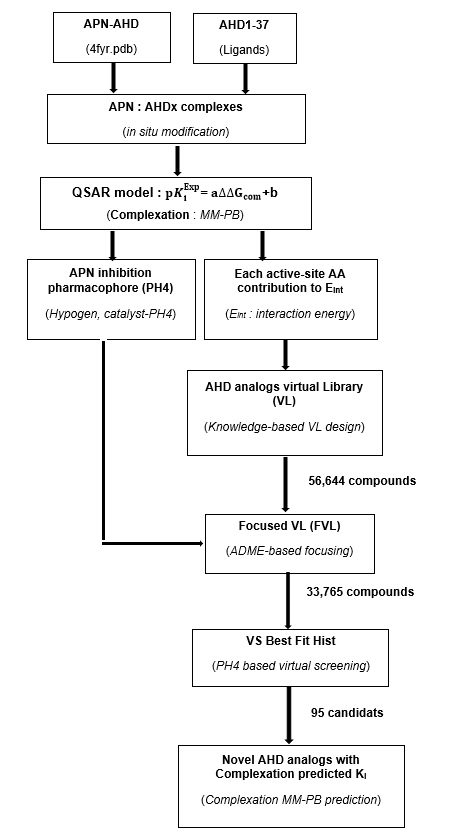
The workflow describing the steps of the entire process of virtual design of novel AHD analogs is presented in scheme 1.

**Training and validation sets**

The training and validation sets of the inhibitors of hydroxamic acid analogs of human APN used in this work come from the literature [39]. Their Kiexp covers a very wide range (4.5 ≤ Kipre ≤ 4,420 nM), more than four orders of magnitude, suitable for a reliable QSAR model. Out of a total of 46 compounds, 37 were used for the training set (TS) and 9 for the validation set (VS) were taken from the reference [39].

**Model building**

Three-dimensional (3D) molecular models of free inhibitors (I), free APN enzyme (E) and enzyme-inhibitor complexes (E:I), were constructed from the high resolution crystal structure (1.91 Å) of a reference complex containing the inhibitor compound AHD1 (PDB code: 4FYR) [39] using the graphical interface available in the molecular modeling program Insight-II [40] and Discovery studio 2.5 [41].



**Scheme 1.** Workflow describing the multistep approach to virtual design novel AHD analogs with higher predicted potency against APN.

**Molecular mechanics**

Modeling of the AHD and PL ligand complexes was carried out by molecular mechanics using the CFF force field [42] as described previously [43].

**Conformational research**

The conformations of the free inhibitors were derived from their bound conformations in the PL complexes by gradual relaxation to the nearest local energy minimum, as previously described [43].

**Gibbs Free Energies Solvation**

Ligand-receptor interactions take place in a solvent, which contributes to the binding process through hydrogen bonding and solvation phenomena. However, the electrostatic component of the Gibbs free energy (GFE) incorporating the effects of the ionic force through solving the nonlinear Poisson-Boltzmann equation [] was calculated by the Delphi module of Discovery Studio 2.5 [41] as described previously [43].

**Calculation of bond Affinity and QSAR model**

The calculation of binding affinity expressed as GFE complexation has been described in detail earlier [43].

**Interaction energy**

The CFF force field was used to calculate the interaction energy (Eint) between the enzyme residues and the inhibitor, as previously reported [43].

**Generation of pharmacophores**

Discovery Studio's 3D-QSAR (PH4) pharmacophore generation protocol [41] via its Catalyst HypoGen algorithmic program [] was used to construct the APN inhibition PH4 as described previously [43].

**ADME properties**

The pharmacokinetic profile of AHDs was calculated by the QikProp program [] as reported earlier [43].

**Virtual library generation**

The generation of the virtual library was carried out as described in ref. [43].

**ADME based library**

The orientation of the virtual library was made using numerous selection criteria as described previously [43].

**Pharmacophore-based library search**

The pharmacophore model (PH4) derived from the bound conformations of AHDs at the APN active site served as a library search tool, as previously described [43].

**Inhibitory power prediction**

The conformer with the best mapping to the PH4 pharmacophore in each group of the targeted library subset was selected for in silico screening by the complexation QSAR model. The ∆∆Gcom calculation of each new selected analog was used to predict the APN inhibitory potency (Kipre) of the targeted AHD analog virtual library by inserting this parameter into the target-specific scoring function given in equation (1) parameterized using the AHD inhibitor training set complexation QSAR model [39].

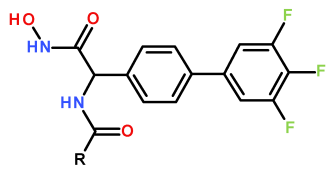
pKipre = −log10Kipre = a.∆∆Gcom+ b                (1)

**RESULTS**

**Training and validation sets**

Forty-six (46) AHDs (Table 1) were selected from a series of compounds with experimentally determined properties and coming from the same laboratory [39]. Their experimental inhibitory activities (4.5 ≤ Kipre ≤ 4420 nM) [39] cover a sufficiently wide range of concentrations to build a reliable QSAR model. The ratio between the sizes of the training and validation sets remains a critical point for correct classification but is limited by the number of sets of homologous compounds available in the literature []. In this study, a training set of 37 AHDs and a validation set of another 9 AHDs (Table 1) were created using the appropriate module of Discovery Studio 2.5 [41].

**Table 1**.Trainingset (AHD1-37) and validation set (VAHD1-9) APN inhibitors [39] used in the elaboration of QSAR model of inhibitor binding. The R group is numbered in the column of the Table as R group index.



|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Training set | AHD1 | AHD2 | AHD3 | AHD4 | AHD5 | AHD6 | AHD7 | AHD8 | |
| R | https://lh6.googleusercontent.com/oCTusNkfYmYkt94VcO3XpG1F2Mlg85QlgomtqA2JdUYvoJZscnJhxh7qAuWrIhWUuyYWHk7GdZvwbt6h4zxJIuIQjRd4hc2xZZgGZWX7b-_YUk5uxkTnquEUpPL33UTXTP6TrhQcNyJJpf2bO8JZ7A | https://lh6.googleusercontent.com/YQKUGKrF25RnDc0YDaq-X-UeGwrTwC4GvCw4kW-cz9kPi4DnJ7iO0awVrej0iFdRxFimr72Yc8S6mD6CBLs0d5hP6bfWc0vmcS8Wfhe31mtW9A6Lv-RvYSG3T7rGUx5iFjQEMSKxYPy1krgvur4L0A | https://lh5.googleusercontent.com/MxveBJbfCsugBLxx5FgtO2JqgG4nkwWQtDvXgSFfmrRcHP6Jeij0JGhdAzz995-XwtA9tgsfUgyyt8wl3Ow0F75Ia6aphewUPIk5vY5y0fTB3c4tb8bZDQ8sk1E0gHVs-E4e8E1WUb0uD7lvR_th_A | https://lh3.googleusercontent.com/ZRiHUCSC3U-kEOULz7sUuqGQBPwxa4P2LRHQlTn84ESFEIQfIxgFBo0R9jpaGdfi_uArZY02LUYZyxL0fUYbPRaI0LVgqk97wEbVC3oT1lOv_PKqBG0DJDusC5O34IzoxZi-DLc4YOtGS6AkiAmlpw | https://lh5.googleusercontent.com/pqtw7i9vVXQ8v4fhDRsroFytpOB94vf2kYNtOouRWNxj_zPuaC8oI7Ia8x6Acjj4FOh3IRf3Y-_WsWMTDLUWb0SRj9qYdbqjHQR8SAWeDHMTBCGSK0dQbGNecKOsI2mABs4Nxx5utbEa70e0205n_w | https://lh4.googleusercontent.com/CABKAyFw1u_vlIHPLIh07oH8pOwsBZEEpuiiY2KRegmOgbxOWp1wMAiIcwd7ZlppLlE4I-Cpr0u-3ZwDitNieQCrla2_yc8wjNGMgolXhv6vYXFDlAEMBDi0hZ0bKEAgTBBBZ3TM1ylKF6S_tYanLA | https://lh3.googleusercontent.com/0Un2tmCofji0RBwbMAF_uS0TwW17X9CILswXnboO1Vy7MI45EV6cYTZFu3JFp5H37Xmrbi57NMy8ureldQXaTnZRjcK_D0BQNOp8gfeltc8GrwNtmvt3CZO7lpINzJ9mc7WFBqqjR7xBkYx1iDCXrg | https://lh4.googleusercontent.com/gI3_WLQpxKo6kN4pkxaIqzKWDB9mLN2RBD37NJlGQGPgOpnhWHLKssZm5TtoHYpW5an17n3IDXZyD2KIN-jTSJC4FP9rCIU0fdkt55hFmzGkbf0yalszq1o7flTwPH_1Zt9Tgkn-XFR4-FxI742aCQ | |
| Kiexp (nM) | 4.5 | 8.2 | 19.2 | 23.4 | 29.1 | 37.3 | 43.1 | 49.1 | |
| Training set | AHD9 | AHD10 | AHD11 | AHD12 | AHD13 | AHD14 | AHD15 | AHD16 | |
| R | https://lh6.googleusercontent.com/uU6Uxyh9UvmZMz3EmZuQT-qYhvP4C1CNDVi0Dufqd_PyabWETF7p637uJeZ1DwnKUguA28_1JYn9nRdFRetr-Kxg9xCyB-rF0dtHduPyUJ71HXFsFl2lFQSoFppbagBknDaAOZwjrHS9HHzsaZsucQ | https://lh6.googleusercontent.com/FPNNb1lAjg7vkeN6wNIwO9_ABkaZo_SZ4KZ9hjMX646_pHJuFMBZj78DSVey70ELfF8e-B3o6zYMIaKAq7f6nYgaKu8FQwj7bEe1Hj2i7ysfZBkZ1lZy8umqhKt33GzwCrT1GCdbHnj8Ikf9GQImPQ | https://lh6.googleusercontent.com/lj6H2D4u3nSJvHiAjjw2h5o2tL9f9MRiQ8X9xmMuPqThVTqRVioIPf_xfnQ2FFaofZcXfNWnjLpvCcEqFUbEWJV2t2bTgyrRlXinV-REUDaFWRGLKP5R483K6W5OAABfMIdJzljflTF3UzGkKxVI-w | https://lh5.googleusercontent.com/k0N491P2WMLUdcm_KMqggsxWv1b83qdlMxe8WR1JO2324UWieY8k1qWMI6SiJAPmkMlRUd8ZgOyv_chL6tjr-JcU7jkI6piKfeJ-EtmLRPAnDtM9DAn_b4DqAYP1ueH4uKST9yi9Hy8P-gIjCmwrhg | https://lh3.googleusercontent.com/oRI-Bh2hQJva5eui-xSaWKEAY9iMQlG407rTaatd_bKvn5qTYKTn2cP8z02eXoVNwaL_rqeF_-_HtMP0x_fVEcVRHLJby8rfcuf_fjTOkxf1MK9luRA4go6b0oEhMeJIBWuxlbFYVE4MDR8_iQSIqw | https://lh5.googleusercontent.com/jdB6QmxOmGPB6-F7Fxa5YXv-boaV8jKa38BjOKizK3E3qas6Lq9XXlJhmubxOsxnlPZP_Dwi81g8cfd8G6Lno22TC9KeY4rey3-Ue8vi-45lwhPI5n_AGR5ZooUUSQ-m9N2At_J07JannJgiNEP38g | https://lh4.googleusercontent.com/Ki1k6gipLgXZeBJ6LRFQDG8VuazA0IBolwKLg7K1kmirCNo0etbSdvhZyA0z3ghywEg9G_hNxBMpNkPbbpUdhLQkAonvISPjMQd3nUy6HB_uw2e76_kqKA-im93URu2dc-AQa0a-LmvdzThmBAV7lw | https://lh6.googleusercontent.com/GaNG21jJJQ6z2EHWAq0ZXSTjP6IO79M7TDc3A8awvVg7PmWt_d7jW4k_ltEOGz_QUvjicy4b3o3ckLR_JvhdMYWe5pJ4uZdXjcsHD7ecgO-J9SUh6f_LiJPMs1auhqDgserHQBYGemmoxDFqIncUIQ | |
| Kiexp (nM) | 71.2 | 82.1 | 111 | 119 | 131 | 137 | 138 | 156 | |
| Training set | AHD17 | AHD18 | AHD19 | AHD20 | AHD21 | AHD22 | AHD23 | AHD24 | |
| R | https://lh3.googleusercontent.com/7l8WXOW1kLidz0Idf3yt2RPrVJoT7c03dAeWuWeWQ5VbLwfXIBI8zvE-gFM7A6kPQLTBsCSM5PDhIN1CMT824CpXDkMv1MJ5B2m_rG3Wpq2cGlZTZUZW-dpm8P4YNjonpY33fCfj32kdnY38RUqAbg | https://lh6.googleusercontent.com/yUBv84Q0SsoZj0qnkFlTMmx0edmqKG7BhWiNt1LCZIRVOILKB_ePbRwjxrqkuFZSdBUwHS6BDCY3nJCF_20-vR_yWIY6d_oT65B7S4NvuQAgjFQCiC2QwXqUnG8hbo9UlKc2KbdmV5Jn3d9e35VDvg | https://lh6.googleusercontent.com/qdvc3bb2rEHFaVeLYHxM2cR_xk5rT4_tMRBfTSofp5htVJR9Su3ychZqqTkRRjS_U-8HxIvNQaRghFUbuT0nVtHwPElNZZWKd_6M8VcQH_v7NKu2uPu4WwMpvdD19ev-stgY6vFPSK1CLK6OSOLelQ | https://lh3.googleusercontent.com/HdHk6G55cjRoy5D6q48_UxYryXPPuG1KU2zggWJDqOaRQilj-cfhPgxHyFWvuQrpYy9iN0KoSx-TVG_DJNS2j8NAUcr-Xc1zzKbvuXOFSnT00AeaYvYDZzdsvFdV38y5E8vKWP0Xr8GZfpaOP5smwA | https://lh6.googleusercontent.com/9DZEirzZAfja1PhhXQKi3O1HAwh5kcXHVX5LLSOzSlICge23-K42ko-hmtmKR94Lzyu-zWYR7v9nMbXavf83eeatbaneh06_gPWpIqCbTft1PoZIDPB-4Z3D32zNGOhT1L_KrfmR9vKKni57dUhUjQ | https://lh5.googleusercontent.com/rVOz37S7H1aIsFA9g_Fz7gETWRqWEKYiXfqMbIMmCcqiIeevIwe1GglKkOy5emiAhVzAw3ZHUc1MQQNUZ70zE7xpYs14hgBtFuuxmwnUHzqrwP7vUqmyXLWPeKTGxQmmtvGwthGukLKldDWma-9MDw | https://lh4.googleusercontent.com/W4qXUipSqe3cWRR50DuP3qMg777HZfiCjnqLifc12uaYXg0PCT3Y6nzmZHFAX-16bHN3hYv3czF8RJtaLPf7g6y9jP8jB0nBBfvpOFgh0-aTRx1Gp23Wwqs3j9aXqSj11tW6IT5RIZLXkjvexvtfzw | https://lh6.googleusercontent.com/MB8PLpPu5SGujnT09kb8VV1FmlICqAYRgMpelW1ybJCUBvCbffTwRX4ESu7QvIXDCykROVPCvQfpr84ar4TNZN2I5jwTmhpTB-uJRdTN4WdW5vQ59yvmP4no5I2l-CmBdjUvDzqnGcGyG4LGBgY7Zg | |
| Kiexp (nM) | 158 | 163 | 172 | 182 | 185 | 188 | 205 | 235 | |
| Training set | AHD25 | AHD26 | AHD27 | AHD28 | AHD29 | AHD30 | AHD31 | AHD32 | |
| R | https://lh3.googleusercontent.com/FOXvE_4mDk81LYn95AxGB9_5z-KWM7yjQDZrGJt4BR7Tk-4MdgTkNrJz7f7yOAYI-4FfQFrJEM2sbq3FSCJlkalGDlODx78wTUMkn2Z0rqHcHw-6NKM02sYCNFZ3Pvrk2zIv_T9kjV70AZcQRCiApA | https://lh3.googleusercontent.com/FOxeaQozWLwmcnsvnhgRDBuIqN8D5cksS3DKsasfvfHaRodMQaVuaZWc8i1iPTIvtis4pKbYLlEAv9EFQbRAe3BIB8oLfjBLqaxPOeGBt3YAO89GhSwKX7i25y4ScPKFt_u3jIEKP4K238khxS1BFQ | https://lh6.googleusercontent.com/pihF7xfZr7iBO2kl4bPUPUprjRy3cPDBHfbs4g8raO0eG4B3GVkx3wSI-iY6tmeRpXKAOqBZK-LIAE2FErm2vo5MSJ6dnJuVX_S69RV9BbeELlteR0JqZ4dcBZ5DtvLmhibnN8bcCuBQx_5Q6MgAKg | https://lh4.googleusercontent.com/ozlh0pyK73ydeQJ1a1GlNLtt_UTWLZqvqC0pIFYWr9dGMyQDI5nJieRpySwmsc_wvlAlKHptq6UCIUPk6qRXmoGNSevd0dmyrP72KvS8ep86JcO9PdykiTw9eOp8FBzLVrehOEmHz-_Yimu4e-d2LQ | https://lh4.googleusercontent.com/Btp_v_ZMXdm4eiu_flCIQFrX3XkaKw3JK0zN6z84MPUE1mYXqNPOwWEuifr3nOFilsIyHQeOuUqAybhJZFxEZTbUzbdbOFeoSiG7p9tOIvYC-hlvC1GgUT99XtLs8nd_tV0thSXYf2zaQWKKnoLdYA | https://lh5.googleusercontent.com/pq-GToOQ3uzwBUq34uXMDHLefrlinOdexP4WO94UYEpP5GRHoD5pjxHILSjVXDHY9vYUDODRexQ9kBRxdwaJBMo45MIcrr9-j5EnhfemOfF_rwLe9hF-CcbVsgkIhXGifP4dl0PQ5ZpIczjjYP_jUg | https://lh5.googleusercontent.com/DO-y0O8Lx7g5UKk2a7zJuodXzuaSwZRtpa22vpQd9WNbFWdA_mKFTPrapObuC8CDUba1Uc1MzlQNroVoN9SOmiZCghBZbk8hVkp9MdW1Z5xHQAe3PYmMkKrC8K7u6zrQ8J2LPm5hsMaZPWSl5tHpow | https://lh3.googleusercontent.com/jR29AcnSZWYINdGBvG4eaxG5eJxZ2ZoZaGViKe4Msrv_lk_rv7dsSCK0GkiMXR1gN20JrkL_CbOrfcsa4sXR88DbWNh085rbJjPsm4ywpZta_0Cm79g56ytgeuy0KtJM1A7YOw9HZ9aaJSgu2PsyCA | |
| Kiexp (nM) | 277 | 348 | 366 | 430 | 442 | 462 | 522 | 631 | |
| Training set | AHD33 | AHD34 | AHD35 | AHD36 | AHD37 |  |  |  | |
| R | https://lh5.googleusercontent.com/Hn1mnE4yyLO8tNomSz3ebi_HJS4bNR501H_YiJZ-L9Pod9-iMhDhoNNaCa0Uhcg2en31QXcNYpYAUGZpaclnfjA7AeKduQGvUhdU9jur07C97IttbxYeQeP6-ps4cwLS6_ZcD1ghTS-_EB8Ud91Z6g | https://lh3.googleusercontent.com/TScL5njiaV-N_4CDDqbpOpxsLFg8xp0gPNytUmlI4hofY7NyIXBhZhL4KHheEQRJ3ad-lYWrCaSEVg5rv1cD54OUGvia-hRMYbF1qbiHqwmapSUJVyL71YB8sMFoT_lQ3KF96kg7UImDXW8a1ojeIw | https://lh6.googleusercontent.com/YDtENMEuo3W5WJVdgeGgOd5tCZ36LulFZe6ak1xe2HsRoNWXU3Fm_CTe803wGlr0tuizVbbxVRhN40AHOO_D-BzaZD3kL4K4rTlYxgmrHhIFRMSkReDoc9Y0ecHy14xzLxO0_Sy7f-9dSqsrIBbP8w | https://lh3.googleusercontent.com/rhTvazeHnRpek1HjA1xBFx0hU8U0QQ5abZ1vILxpWuzvvYNsH5zmKhS66vVW7PuuN_D212QARGeE2OvplSL8zu8hV73CWY58LNleYeh-2AF8CEhQKSi4dgwZ7P_-W8YcDQ6fgRtnccd6Dop0tSnvkQ | https://lh5.googleusercontent.com/YhTJA4OlmByM80DX8L15vdiqniB85EESslU6iZVNX_cde67o5WqZJaDrqOYsUqnms1DRZDbO-01H4MvCqO8EYT95CBUPFNB5pPp7SrqbGZzBnXtyL2a0yl-iID5WbnJ4jbD90oZurpITxuL_8e8Qhw |  |  |  | |
| Kiexp (nM) | 704 | 745 | 919 | 978 | 4420 |  |  |  | |
|  |  |  |  |  |  |  |  |  | |
| Validation set | VAHD1 | VAHD2 | VAHD3 | VAHD4 | VAHD5 | VAHD6 | VAHD7 | VAHD8 | VAHD9 |
| R | https://lh4.googleusercontent.com/53kRSEX9IYV7WJdB-Tr9QZrIgL_L5WUqcpJpi3qZEY-CYXQ1bIle8-Nin0Js0dYsKrZs5B6XS95Agqdp__OmC5J_osBdXbk-Idw5FSzHB4B6DIc-36ZkZadQyJRtHI-RnhAfV2ZrZ53rY0SOnbcpDw | https://lh4.googleusercontent.com/pApJX4ylVBrowG0gw_SFGguwLnsKtM7xDm10fpjRq71njiVABkbwDtjTlUOh9wyeqT4AwQ_urkHafulWQ_GSIzya8ezbNXxm0rdKrneclaeBmSFrf3YVjOb2x0weXaZutgA85oz-hOK5v_SJJyhGFA | **−C(CH3)3** | https://lh5.googleusercontent.com/oqvZG0VNlf7dH7wkkxlCabr_lIYBeWUei2pjq-UyMG4AKpXwmBJclGMm9CZ8lJzKF8MDSzXc30cVJRvNBK96KKwHKNgLpdD9r1YP4ggjQPe8L-6cPUJWveyyZQHHa3WMOzGML1IZszlE2Xy7dHGvtg | https://lh5.googleusercontent.com/ykWB6exPwHyFdgGiOXMv1LLuu6rfwRoxSI12Ud6QSE-lVOaN7GLT8DMbsSR60JEFctWppJ_KBVquDPqlgs7d_YPBOkYeFFLP_FiAdOAM5p8SD85xFBh1T_fFItncVCjVbhiTTn651IkVoBoAIaybWQ | https://lh3.googleusercontent.com/_CpfTIqsWyhywamUCQgYnghLj34cwJO_e9jNHI6SWLMpe3NMQzSTD3lcbWAPhO0fHlDeKwQ1aQsENHlzXI192LS6clweWg0VCxU1nJE20MOWMRsrQhdDcqnkwXEwleADkfoSIOTH0kR3e-fBBmtkCQ | https://lh6.googleusercontent.com/BDraBMa5VqkE03jHChg0_LKutUdCA00GdsjYGSftVrVo81loj1-V7gCsOl0vDgzn0jk47xWTFegEYginLa73R1OUxMuIPIpYxsKCyhqxofScmnMkAzSSCzOqC9Zt7K55RSODo0IJHUcW2x4pmHAU5w | **−CH3** | https://lh6.googleusercontent.com/pyeg8sGkZfHy_B3S8ebB2i2fIAuMxKHlCx_Ii_OTFX6FHUfCUrSMxZ0wpt2d-ITozBESxlCOjzruV9hOaMPFM0lwZxh2TXGmL0Dd2Uiu9HU7XM68QSShcs2xZEP_haEq8Y7mfz9jDyCvdl2Q91SoxQ |
| Kiexp (nM) | 40 | 102 | 118 | 170 | 175 | 240 | 497 | 560 | 604 |

**One-descriptor QSAR model**

Each of the 37 training sets (TS) and 9 validation sets (VS) APN: AHDx complexes (Table 1) was prepared by in situ modification of the crystal structure of the refined model (PDB entry code 4FYR) [39] of the APN: AHD1 complex as described in the Methods section. Additionally, the relative Gibbs free energy (GFE) of APN: AHDx ∆∆Gcom complex formation was calculated for each of the 46 optimized enzyme-inhibitor complexes. Table 1 lists the calculated values of ∆∆Gcom and its components as defined in equation (7), for the TS and VS of hydroxamic acid [39]. The QSAR model explained the variation in the experimental inhibitory powers of AHDs (pKiexp = – log10(Kiexp)) by correlating it with the GFE ∆∆Gcom calculated by linear regression (equation (8), table 2). Furthermore, from this significant correlation obtained in this QSAR relationship, the active bound conformation of AHDs at the APN binding site was determined, leading to the definition of the APN inhibition pharmacophore (PH4). To evaluate the step-by-step understanding of the binding affinity of AHDs toward APN, we first calculated and analyzed the gas-phase complexation enthalpy ∆∆HMM by correlating it with the pKiexp. The validity of this linear correlation (for the statistical data of the regression, see Table 3, equation A) allowed the significance of inhibitor-enzyme interactions (∆∆HMM) when the effect of the solvent and the loss of entropy of the inhibitor upon binding to APN were neglected. This correlation explains approximately 86% of the variation in pKiexp data and highlights the role of enthalpy contribution in ligand binding affinity. Likewise, the subsequent more advanced descriptor, namely the GFE of the APN:AHDx complex formation including all components: ∆∆HMM, ∆∆TSvib and ∆∆Gsol, was evaluated (for statistical data, see Table 3, equation B) reaching a relatively high values (0.94) of the regression coefficient R2 attesting that  structural information derived from 3D models of APN–AHDx complexes should lead to reliable prediction of APN inhibitory potencies for novel AHD analogues (sharing the same binding mode) based on the QSAR B model, Table 3.

**Binding mode of AHDs**

The inhibitors (AHDs) we have used throughout this work are a reported new series of hydroxamic acid obtained by synthesis [39]. Indeed, hydroxamic acids are used as metal ion chelators and the presence of the acid function in their molecular structure makes them particularly important for the inhibition of APN. Active site have been assessed from the X-rays crystal structure analysis of APN (PDB code 4FYR) in complex with one of the most active studied inhibitors in this work (4) [39].

1. **Interaction Energy**

Other key structural information was provided by the interaction energy (IE, ∆Eint) diagram obtained for each training set inhibitor. IE breakdown to contributions from APN active site residue is helpful for the choice of relevant R groups (site S1, S1’) which could improve the binding affinity of AHD analogs to the APN and subsequently enhance the ligand potency. A comparative analysis of computed IE for the training set AHDs (Figure 4) divided into three classes (highest, moderate, and lowest activity) has been carried out to identify the residues for which the contribution to binding affinity could be increased. How-ever, the comparative analysis showed about the same level of IE contributions from active site residues for all three classes of inhibitors and no suggestions of suitable substitutions able to improve the binding affinity as we previously reported for thymine-like inhibitors of APN

**Table 2.** Gibbs free energy (binding affinity) and its components for the training set of *APN* inhibitors AHD1-37 and validation set inhibitors VAHD1-9 [39].

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Training Set a | MW b | ∆∆HMM c | ∆∆Gsol d | ∆∆TSvib e | ∆∆Gcom f | **Kiexp  g** |
| AHD1 | 493 | 0.00 | 0.00 | 0.00 | 0.00 | 4.5 |
| AHD2 | 494 | -0.64 | 1.83 | -0.70 | 1.89 | 8.2 |
| AHD3 | 440 | 2.78 | 1.71 | 1.28 | 3.20 | 19.2 |
| AHD4 | 441 | 1.89 | 2.57 | 1.94 | 2.52 | 23.4 |
| AHD5 | 434 | 2.29 | 3.21 | 0.72 | 4.79 | 29.1 |
| AHD6 | 460 | 5.15 | 2.12 | 2.42 | 4.86 | 37.3 |
| AHD7 | 448 | 5.31 | 2.23 | 2.33 | 5.21 | 43.1 |
| AHD8 | 460 | 4.82 | 3.24 | 1.53 | 6.53 | 49.1 |
| AHD9 | 443 | 5.61 | 0.79 | 0.81 | 5.59 | 71.2 |
| AHD10 | 443 | 5.71 | 1.01 | 0.66 | 6.06 | 82.1 |
| AHD11 | 439 | 5.99 | 2.34 | 2.42 | 5.91 | 111 |
| AHD12 | 474 | 7.88 | 3.35 | 4.22 | 7.00 | 119 |
| AHD13 | 415 | 7.90 | 3.75 | 3.81 | 7.85 | 131 |
| AHD14 | 446 | 8.17 | 2.06 | 2.85 | 7.37 | 137 |
| AHD15 | 448 | 6.20 | 5.20 | 3.46 | 7.94 | 138 |
| AHD16 | 455 | 8.74 | 0.09 | 1.06 | 7.77 | 156 |
| AHD17 | 432 | 6.83 | 3.68 | 2.01 | 8.49 | 158 |
| AHD18 | 487 | 7.20 | 0.11 | 0.29 | 7.02 | 163 |
| AHD19 | 396 | 6.08 | 3.91 | 1.22 | 8.77 | 172 |
| AHD20 | 471 | 8.06 | 1.43 | 2.03 | 7.46 | 182 |
| AHD21 | 457 | 5.99 | 1.55 | -0.80 | 8.34 | 185 |
| AHD22 | 382 | 8.18 | 1.36 | 1.48 | 8.05 | 188 |
| AHD23 | 415 | 5.94 | 5.38 | 3.03 | 8.29 | 205 |
| AHD24 | 430 | 7.42 | 3.83 | 3.80 | 7.45 | 235 |
| AHD25 | 471 | 7.24 | 2.91 | 1.51 | 8.65 | 277 |
| AHD26 | 397 | 8.93 | 4.13 | 2.24 | 10.82 | 348 |
| AHD27 | 416 | 7.98 | 3.76 | 2.41 | 9.33 | 366 |
| AHD28 | 432 | 8.83 | 5.39 | 2.89 | 11.33 | 430 |
| AHD29 | 432 | 7.20 | 3.80 | 0.90 | 10.10 | 442 |
| AHD30 | 430 | 7.16 | 6.76 | 2.47 | 11.45 | 462 |
| AHD31 | 400 | 11.12 | 1.37 | 2.07 | 10.42 | 522 |
| AHD32 | 485 | 8.97 | 3.39 | 2.91 | 9.45 | 631 |
| AHD33 | 418 | 11.93 | 0.28 | 1.02 | 11.19 | 704 |
| AHD34 | 430 | 10.19 | 3.44 | 2.05 | 11.58 | 745 |
| AHD35 | 418 | 10.86 | 2.05 | 0.89 | 12.02 | 919 |
| AHD36 | 444 | 10.99 | 2.82 | 3.28 | 10.53 | 978 |
| AHD37 | 414 | 12.70 | 3.49 | -4.81 | 15.08 | 4420 |
| Validation set a | MW b | ∆∆HMM c | ∆∆Gsol d | ∆∆TSvib  e | ∆∆Gcom  f | pkipre/ pkiexp h |
| VAHD1 | 434 | 5.31 | 1.87 | 0.96 | 6.23 | 0.96 |
| VAHD2 | 416 | 5.20 | 1.77 | 1.28 | 5.70 | 1.03 |
| VAHD3 | 380 | 11.20 | 3.50 | 8.46 | 6.24 | 1.02 |
| VAHD4 | 442 | 5.69 | 4.36 | 2.84 | 7.20 | 1.02 |
| VAHD5 | 460 | 8.87 | 0.67 | 2.70 | 6.84 | 1.03 |
| VAHD6 | 479 | 7.63 | 2.76 | 0.64 | 9.75 | 0.97 |
| VAHD7 | 395 | 8.87 | 3.17 | 2.86 | 9.18 | 1.04 |
| VAHD8 | 338 | 12.30 | -2.18 | 2.89 | 7.23 | 1.11 |
| VAHD9 | 457 | 10.94 | -0.32 | 2.11 | 8.50 | 1.07 |

a for the chemical structures of the training set of inhibitors see Table 1; b Mw (g/mol) is the molar mass of inhibitors; c ∆∆HMM (kcal/mol) is the relative enthalpic contribution to the GFE change related to E-I complex formation derived by MM;∆∆HMM ≈[EMM{E-Ix} − EMM{Ix}] − [EMM{E-Iref} − EMM{Iref}], Iref is the reference inhibitor HDA1; d∆∆Gsol (kcal/mol) is the relative solvent effect contribution to the GFE change of E-I complex formation: ∆∆Gsol = [Gsol{E-Ix} − Gsol{Ix}] − [Gsol{E-Iref} − Gsol{Iref}]; e −∆∆TSvib (kcal/mol) is the relative entropic contribution of inhibitor to the GFE of E-Ix complex formation: ∆∆TSvib = [TSvib{Ix}E − TSvib{Ix}] − [TSvib{Iref}E − TSvib{Iref}]; f∆∆Gcom (kcal/mol) is the overall relative GFE change of E-Ix complex formation: ∆∆Gcom ≈∆∆HMM + ∆∆Gsol − ∆∆TSvib; g Kiexp (nM) is the experimental inhibitory concentration of *APN* obtained from ref. [39] ; h ratio of predicted and experimental half-maximal inhibition concentrations pKipre/pKiexp(pKipre = −log10Kipre) was predicted from computed ∆∆Gcom using the regression equation for APN shown in Table 3, B.

**Table 3.** Analysis of computed binding affinities ΔΔGcom, its enthalpic component ΔΔHMM, and experimental inhibitory concentrations pKiexp = −log10Kiexp of HDAs towards APN [39]

|  |  |  |
| --- | --- | --- |
| **Statistical Data of Linear Regression** | (A) | (B) |
| pKiexp= -0.1866×∆∆HMM + 8.0965 (A) | | |
| pKiexp= -0.1901×∆∆Gcom + 8.2886 (B) | | |
| Number of compound  n | 37 | 37 |
| Squared correlation coefficient of regression R2 | 0.86 | 0.94 |
| LOO cross-validated squared correlation coefficient R2xv | 0.86 | 0.94 |
| Standard error of regression σ | 0.23 | 0.14 |
| Statistical significance of regression. Fisher F-test | 224.1 | 584.3 |
| Level of statistical significance (%) | >95 | >95 |
| Range of activities Kiexp [nM] | 4.5 – 4,420 | |

The statistical data confirmed validity of the correlation Equations (A) and (B) plotted on Figure 3. The ratio Kipre/Kiexp ≈1 (the Kipre values were estimated using correlation Equation B, Table 3) calculated for the validation set VAHD1-9 documents the substantial predictive power of the complexation QSAR model from Table 2. Thus, the regression Equation B (Table 3) and computed ∆∆Gcom GFEs can be used for prediction of inhibitory potencies Kipre against APN for novel AHD analogs, provided they share the same binding mode as the training set hydroxamic acid AHD1-37.

|  |
| --- |
| https://lh4.googleusercontent.com/NdzG-JvL3ZF7gtIC1NbMuIpTCUl7pCkyeFv3PcgcjB7i8Sk4fsFopQrO9vHCx0LquIENs6nltgoYT6Hr5gP86arPT3UnHNJKX6Q4n8eZt6adBHfpiH7N_0n8gKXwdU6WqG7QF4CzuIDNYngL6x-ERg |
| https://lh3.googleusercontent.com/02jd_KW-C1WMsse1np1CSS6Tvl2Tg8QH9gX9LnEbYh6cuYgurhIX0o2Xn12xdOP7c-FvT0frDbGQKd1sSqB_HaipWorI0czaPDbo1RCsVNA03DuS6pDMZ5AKjNEbg5cEPDGM6AD6mV9DjmBSmn4Zkg |
| **Figure 3. (**Top) plot of correlation equation between pKiexp and relative enthalpic contribution to the GFE (∆∆HMM [kcal.mol-1]). (Bottom) similar plot for relative complexation Gibbs free energies of the APN-AHD complex formation ∆∆Gcom [kcal.mol-1] of the training set [39]. The validation set data points are shown in red color |

|  |
| --- |
| https://lh4.googleusercontent.com/G2BZefpajU1ypsRcjkBIRJh_G8UTWBjf3gd8SMocpWfV_QOaiJsevuEBDOzsgPmFmEiCcNIyBYiV5U56shoNVYEi-e1yLNHeICLv5EmgkVc3p0KvIkKPD1runigPrdmGJBo2own5lRtUwiWzBCkPTQ |
| https://lh5.googleusercontent.com/q2ct_EQSKTL8UM2M50J9lBQnn9vwn9QvTsgYVwDrSRT0lMJ7w31LplJo_0EG7RWytoOcKrO8HSQ02L0kiUfaR6zYDJHd9603rw2MUtuorqTLfOffbAajOYKdhxFF5q9OR4bzmkoW2D6_QlaehopHQw |
| **Figure 4.** (Top) 2D schematic interaction diagram of the most potent inhibitor AHD at the active site of APN and (Bottom) 3D schematic interaction of AHD1 at the enzyme active site |

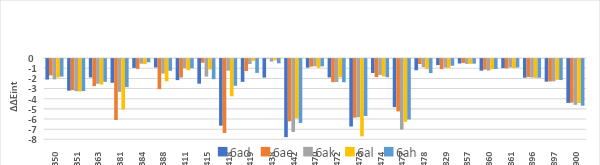
**3D-QSAR Pharmacophore Model**

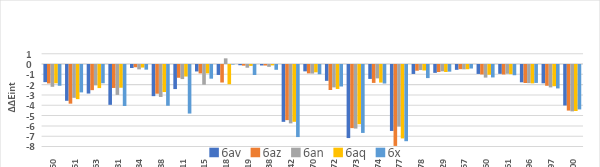
**Generation and Validation of Pharmacophore**

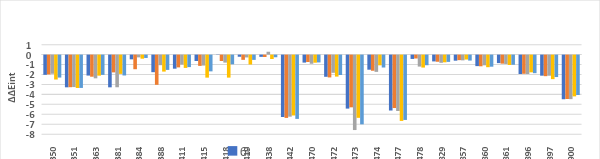
APN inhibition 3D-QSAR pharmacophore was generated from the active conformation of 37 TS AHD1-37 and evaluated by 9 VS VAHD1-9 covering a large range of experimental activity (4.5 – 4420 nM) spanning almost three orders of magnitude. The generation process is divided into three main steps: (i) the constructive step, (ii) the subtractive step, and (iii) the optimization step [39] as described earlier [43].

Accordingly, none of the training set AHDx was inactive and no starting PH4 features were removed. Finally, during the optimization phase, the score of the pharmacophoric hypotheses was improved. Hypotheses were scored according to errors in activity estimates from regression and complexity via a simulated annealing approach. At the end of the optimization, the top scoring 10 unique pharmacophore hypotheses were kept, all displaying five-point features. The cost values, correlation coefficients, root-mean square deviation (RMSD) values, the pharmacophore features, and the max-fit value of the top 10 ranked hypotheses (Hypo1-Hypo10) are listed in Table 4. They were selected based on significant statistical parameters, such as high correlation coefficient, low total cost, and low RMSD

.







**Fig. 5**. Molecular Mechanics intermolecular interaction energy Eint breakdown to residue contributions in [kcal.mol-1]: (Top) the most active inhibitors AHD1 (4.5 nM) – AHD5 (29.1 nM), (Middle) moderately active inhibitors AHD15 (138 nM) – AHD19 (172 nM), (Bottom) less active inhibitors AHD33 (704 nM) – AHD37 (4420 nM), Table 2 [39]

**Table 4.** Parameters of 10 generated PH4 pharmacophoric hypotheses for APN inhibitor after Cat-Scramble validation procedure (49 scrambled runs for each hypothesis at the selected level of confidence of 98%).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Hypothesis** | **RSMD a** | **R2 b** | **Total Cost c** | **Costs Difference d** | **Closest Random e** |
| Hypo 1 | 6.641 | 0.89 | 868.1 | 2995.3 | 1459.4 |
| Hypo 2 | 9.523 | 0.75 | 1736.1 | 2127.2 | 1459.9 |
| Hypo 3 | 9.689 | 0.74 | 1793.6 | 2069.8 | 1852.9 |
| Hypo 4 | 9.728 | 0.74 | 1805,6 | 2057.7 | 1959.5 |
| Hypo 5 | 9.748 | 0.73 | 1814.1 | 2049.3 | 1986.6 |
| Hypo 6 | 9.767 | 0.73 | 1821.1 | 2042.3 | 2003.2 |
| Hypo 7 | 9,790 | 0.73 | 1828.8 | 2034.6 | 2043.6 |
| Hypo 8 | 9.823 | 0.73 | 1842,5 | 2020.8 | 2050.8 |
| Hypo 9 | 9.826 | 0.73 | 1843.7 | 2019.6 | 2071.5 |
| Hypo 10 | 9.842 | 0.73 | 1844.8 | 2018.8 | 2114.8 |
| Fixed Cost | 0 | 0 | 49.63 |  |  |
| Null Cost | 14.387 | 0 | 3863.34 |  |  |

Configuration cost = **14.50.**

**a** root mean squared deviation; **b** squared correlation coefficient; **c** overall cost parameter of PH4 pharmacophore; **d** cost difference between Null cost and hypothesis total cost; **e** lowest cost from 49 scrambled runs at a selected level of confidence of 98%.

The generated pharmacophore models were then assessed for their reliability based on the calculated cost parameters ranging from 868.06 (Hypo1) to 1844.82 (Hypo10). The relatively small gap between the highest and lowest cost parameter corresponds well with the homogeneity of the generated hypotheses and consistency of the TS of AHDx. For this PH4 model, the fixed cost (49.63) is lower than the null cost (3863.34) by a difference Δ = 3813.71. As reported earlier [39], this difference is a major quality indicator of the PH4 predictability (Δ > 70 corresponds to an excellent chance or a probability higher than 90% that the model represents a true correlation [46]. To be statistically significant, a hypothesis has to be as close as possible to the fixed cost and as far as possible from the null cost. For the set of 10 hypotheses, the difference Δ ≥ 1844.82 which attests to the high quality of the pharmacophore model. The standard indicators such as the RMSD between the hypotheses ranged from 6.64 to 9.84, and the squared correlation coefficient (R2) falls to an interval from 0.89 to 0.73. The first PH4 hypothesis with the total costs (868.06) and best RMSD and R2 was retained for further analysis. The statistical data for the set of hypotheses (costs, RMSD, R2) are listed in Table 4. The configuration cost (14.50 for all hypotheses) far below 49 confirms this pharmacophore as a reasonable one. The evaluation of Hypo 1 is the mapping of the best active training set AHD1 (Figure 4 (D)) displaying the geometry of the Hypo1 pharmacophore of APN activation. The regression equation for pKiexp vs. pKipre estimated from Hypo1: pKiexp = 1.0006× pKipre + 0.0028 (n = 37. R2 = 0.79. R2xv = 0.78. F-test = 130.03. σ = 0.28, α > 95%)  is also plotted on Figure 4 (E). Therefore, the PH4 is good potentially to choice the new AHD analogs.

|  |  |
| --- | --- |
| https://lh3.googleusercontent.com/DHxXflTJcqqzwlpWlgEZBAbL--Yw-z6CkAAVv9cI9-EoRI97dR6zENGg48oYX4OALgcDDl739isysq_-kU5610_ithSUxeeZXJGqKdRHYaYddFp2zXi1IH9Ksfx3-lq4lwaCQXW11G3iaRpizYn33g | https://lh3.googleusercontent.com/NFsMFIKOnWVfsUrhkQVvFxpHThuE5Yz9vr_cNrOD1ywvjRCLr0DelGuctb2BXnXAG5VHnDBYeoAMBIyjf_fxizErOtIlcwMQszF7bGcES11NI7_jTgBmmzYZBCABiXt26x5n5KaEIaNXKO4ApI6ddQ |
| https://lh3.googleusercontent.com/bWTCbTwiFwZwNxIPDfKOV5zkawLAIOKDsvtVo7jA0Iy6pfPb8dj5pQi69HRaqLTGP7sSgnllqZy0LKsrfwUHNp1k34nJ20dnKOtsvDQCuOMmzUyPd3nIsx-cZfReyfu4c4M-VBO21Oo7bqCPdJ-JUQ | https://lh3.googleusercontent.com/GT0NHgAzuGiqGcJrmyY2E9hp-Dl9hwjKc3sem4Fb0RPXFxZpy9EY2T57O6SYIQ0crS0D7_SQFrs5BVgzIge6rJ4ZWP0XD7T86ZjD9B6rsbZuwElIAYEWKuTv9N-5-36H6r3hydakZ6M0XtS8SXMbxQ |
| https://lh3.googleusercontent.com/LGC41GIOAE2_kGjQFDNOOuofwDnlk-3hxP_c8p41f1KMBN6Lp76jx_KAMwZ3vYYgn91orTYiAtOTRvp-JqUfn-qkRchjvJhNVfmjl1NLk_Lo-UwtsfKPqWAVIfIBazrT0QRVsj7PXiF-_k9AOQcFBg | |
| **Figure 6.** (Top left) Features coordinates of centers, (Top right) Distances between centers, (Middle left) angles between centers of pharmacophoric features, (Middle right) mapping of pharmacophore of APN inhibitor with the most potent molecule AHD1. Feature legend: HYD = Hydrophobic (cyan), HBA = Hydrogen bond Acceptor (green), HBD = Hydrogen bond Donor (pink). (Bottom) Correlation plot of experimental vs. predicted inhibitory activity. | |

**Virtual Screening**

In silico screening of a virtual (combinatorial) library can lead to hit identification as it was shown in our previous works on inhibitors design [48] [49] [50] [51] [52] [53].

**Virtual Library**

An initial Virtual library (VL) was generated by substitutions at positions for R1 and R2 (see Table 5) on the scaffold. During the virtual library enumeration, the R-groups listed in Table 5 Were attached to in positions R1 and R2 of the AHD scaffold to form a combinatorial library of the size: R1 × R2 = 252 252 = 56 644 analogs. All analogs are matching the substitution pattern of the best inhibitor AHD1. This AHDs analogs library was generated from fragments (chemicals) listed in databases of available chemicals [54]. To design a more target library of reduced size and increased content of drug-like molecules, we have introduced a set of filters and penalties such as the Lipinski rule-of-five (Mw > 500 g/mol) [55], which helped to select a smaller number of suitable AHDs that could be submitted to in silico screening.

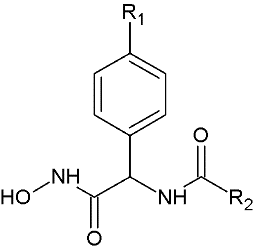
***In silico* Screening of Library of AHDs**

The focused library of 56 644 analogs was further screening for molecular structures matching the 3D-PH4 pharmacophore model Hypo1 of APN inhibition. 95 AHDs mapped to at last 4 features of the pharmacophore according to the so-called similarity-property principle (SPP) according to which structurally similar compounds exhibit similar biological effects against the same target. These best fitting analogs (PH4 hits) then underwent complexation QSAR model screening. The computed GFE of APN-AHDs complex formation, their components, and predicted half-maximal inhibitory concentrations Kipre calculated from correlation Equation B (Table 3) are listed in Table 6).

**Novel AHD analogs**

The design of virtual library of novel analogs was guided by structural information retrieved from the AHDs active conformation and the pharmacophore model, used for the selection of appropriate substituents. The hydrophobic feature of PH4 at the position R1 shows clearly the type of group to be oriented towards the hydrophobic pocket S1. The analysis of frequency of occurrence of R-group during the selection of appropriate surrogates for two points of attachment: R1-group and R2- group shows that the frequency of occurrence of groups R1 and R2 among the best resulting from PH4 (Fig. 8) is as follows: for the large hydrophobic pocket S1’ filling R2-groups, **151**: 4-(5-methyl-3-furyl)phenyl, **159**: 4-(2-sulfanylcyclopenta-2,4-dien-1-yl)phenyl, **250**: 2-sulfa-nylacetyl)amino, **115**: 4-sulfanyl-5-(sulfanylmethyl)-pyrazol-1-yl, 57: 3-methanimidoylbenzenethiol, **242**: 4-sulfamoylphenyl, **158**: 4-[3,4-bis(sulfanyl)cyclopenta-2,4-dien-1-yl]phe-nyl with occurrences of 16, 8, 5, 7, 7, 5 respectively are the most represented while **48**: (3-sulfanylphenyl)for-mate with 2 occurrences, appears in the highest potency AHD analogs. In the smaller hydrophobic pocket S1, filling R1-groups **1**: cyclopenta-2,4-diene-1-carbonyl, **17**: amino(cyclopenta-2,4-dien-1-yl)methyl, **106**: 5-fluoro-pyrazol-1-yl with occurrences of 5, 6, 5 is the most represented while **243**: benzenesulfonyl and **126**: pyrimidin-4-yl occurrences 1 and 2 appearing in  the top 4 highest potency AHD analogs. The best analogs from these most commonly used substituents (R1-group: R2-group) are: 243- 242 (Kipre = 0.05 nM); 126-242 (Kipre = 0.07 nM); 126-158 (Kipre = 0.09 nM); 17-48 (Kipre = 0.13 nM). Branching larger aliphatic moieties in the R2 position for better filling the large S1’ pocket and conserving HB interactions and keeping almost the size in R1 position for the smaller hydrophobic pocket S1 of the AHD analogs contributed strongly to an overall improvement in the inhibitory activity against human M1 aminopeptidase (APN). This relates to the inhibitory potency of the best proposed new analogs.

**Table 5**. R1 and R2-groups (fragments, building blocks, substituents) used in the design of the initial diversity virtual combinatorial library.



|  |  |  | **R-groups** |  |  |
| --- | --- | --- | --- | --- | --- |
| 1 | cyclopenta-2,4-diene-1-carbonyl | 2 | 2-methylcyclopenta-2,4-diene-1-carbonyl | 3 | 2-fluorocyclopenta-2,4-diene-1-carbonyl |
| 4 | 2-aminocyclopenta-2,4-diene-1-carbonyl | 5 | 2-sulfanylcyclopenta-2,4-diene-1-carbonyl | 6 | 3-sulfanylcyclopenta-2,4-diene-1-carbonyl |
| 7 | 2,3-bis(sulfanyl)cyclopenta-2,4-diene-1-carbonyl | 8 | 2-chlorocyclopenta-2,4-diene-1-carbonyl | 9 | 3-chlorocyclopenta-2,4-diene-1-carbonyl |
| 10 | 2,3-dichlorocyclopenta-2,4-diene-1-carbonyl | 11 | 3-bromocyclopenta-2,4-diene-1-carbonyl | 12 | 2-bromocyclopenta-2,4-diene-1-carbonyl |
| 13 | 2,3-dibromocyclopenta-2,4-diene-1-carbonyl | 14 | 2-iodocyclopenta-2,4-diene-1-carbonyl | 15 | 3-iodocyclopenta-2,4-diene-1-carbonyl |
| 16 | 2,3-diiodocyclopenta-2,4-diene-1-carbonyl | 17 | amino(cyclopenta-2,4-dien-1-yl)methyl | 18 | amino-(2-fluorocyclopenta-2,4-dien-1-yl)methyl |
| 19 | amino-(2,3-difluorocyclopenta-2,4-dien-1-yl)methyl | 20 | amino-(2-sulfanylcyclopenta-2,4-dien-1-yl)methyl | 21 | amino-[2,3-bis(sulfanyl)cyclopenta-2,4-dien-1-yl)methyl |
| 22 | 2,3-bis(sulfanyl)cyclopenta-2,4-dien-1-yl)methyl-(sulfanylamino)methyl | 23 | sulfanylamino-(2-sulfanyl)cyclopenta-2,4-dien-1-yl)methyl | 24 | sulfanylamino-(3-sulfanyl)cyclopenta-2,4-dien-1-yl)methyl |
| 25 | 3-fluorocyclopenta-2,4-dien-1-yl-(sulfanyamino)methyl | 26 | 2-fluorocyclopenta-2,4-dien-1-yl-(sulfanyamino)methyl | 27 | 2,3-difluorocyclopenta-2,4-dien-1-yl-(sulfanyamino)methyl |
| 28 | 2,3-bis(sulfanyl)cyclopenta-2,4-dien-1-yl)methyl-(fluoroamino)methyl | 29 | fluoroamino-(2-sulfanylcyclopenta-2,4-dien-1-yl)methyl | 30 | fluoroamino-(3-sulfanylcyclopenta-2,4-dien-1-yl)methyl |
| 31 | fluoroamino-(3-fluorocyclopenta-2,4-dien-1-yl)methyl | 32 | 2,3-difluorocyclopenta-2,4-dien-1-yl-(fluoroamino)methyl | 33 | 2,3-dichlorocyclopenta-2,4-dien-1-yl-(fluoroamino)methyl |
| 34 | 2-chlorocyclopenta-2,4-dien-1-yl-(fluoroamino)methyl | 35 | 3-chlorocyclopenta-2,4-dien-1-yl-(fluoroamino)methyl | 36 | 3-bromocyclopenta-2,4-dien-1-yl-(fluoroamino)methyl |
| 37 | 2,3-dibromocyclopenta-2,4-dien-1-yl-(fluoroamino)methyl | 38 | 2-bromocyclopenta-2,4-dien-1-yl-(fluoroamino)methyl | 39 | 2-carbamoylcyclopenta-2,4-dien-1-yl-(fluoroamino)methyl |
| 40 | 3-carbamoylcyclopenta-2,4-dien-1-yl-(fluoroamino)methyl | 41 | 2-carbamoyl-3-fluoro-cyclopenta-2,4-dien-1-yl-(fluoroamino)methyl | 42 | 2-carbamoyl-3-chloro-cyclopenta-2,4-dien-1-yl-(fluoroamino)methyl |
| 43 | 3-amino-2-carbamoyl--cyclopenta-2,4-dien-1-yl-(fluoroamino)methyl | 44 | (2-carbamoylphenyl) formate | 45 | (3-carbamoylphenyl) formate |
| 46 | (4-carbamoylphenyl) formate | 47 | (2-sulfanylphenyl) formate | 48 | (3-sulfanylphenyl) formate |
| 49 | (4-sulfanylphenyl) formate | 50 | [2,3-bis(sulfanyl)phenyl] formate | 51 | 2-methanimidoylbenzamide |
| 52 | Phenylmethanimine | 53 | 3-methanimidoylbenzamide | 54 | 4-methanimidoylbenzamide |
| 55 | 2-methanimidoylbenzenethiol | 56 | 3-methanimidoylbenzene-1,2-dithiol | 57 | 3-methanimidoylbenzenethiol |
| 58 | 4-methanimidoylbenzenethiol | 59 | (Z)-N-fluoro-1-(2-fluorophenyl)methanimine | 60 | (Z)-N-fluoro-1-(3-fluorophenyl)methanimine |
| 61 | (Z)-1-(3-bromophenyl)-N-fluoro-methanimine | 62 | (Z)-1-(2-bromophenyl)-N-fluoro-methanimine | 63 | (Z)-1-(2-chlorophenyl)-N-fluoro-methanimine |
| 64 | (Z)-1-(3-chlorophenyl)-N-fluoro-methanimine | 65 | (Z)-N-bromo-1-(3-chlorophenyl)methanimine | 66 | (Z)-N-bromo-1-(3-bromophenyl)methanimine |
| 67 | (Z)-N-chloro-1-(3-chlorophenyl)methanimine | 68 | (Z)-N-chloro-1-(2-chlorophenyl)methanimine | 69 | o-tolylmethanimine |
| 70 | [2-(trifluoromethyl)phenyl]methanimine | 71 | [3-(trifluoromethyl)phenyl]methanimine | 72 | 3-methylbenzaldehyde |
| 73 | 2-formylbenzamide | 74 | 4-formylbenzamide | 75 | 2-sulfanylbenzaldehyde |
| 76 | 2,3-bis(sulfanyl)benzaldehyde | 77 | 3-sulfanylbenzaldehyde | 78 | 4-sulfanylbenzaldehyde |
| 79 | 2-methylbenzaldehyde | 80 | 2-(trifluoromethyl)benzaldehyde | 81 | 3-(trifluoromethyl)benzaldehyde |
| 82 | 2-fluorobenzaldehyde | 83 | 2-(aminomethyl)-6-bromo-benzamide | 84 | formamide |
| 85 | 4-chloropyrazol-1-yl | 86 | 4,5-dichloropyrazol-1-yl | 87 | 5-chloropyrazol-1-yl |
| 88 | 3-chloropyrazol-1-yl | 89 | 3-bromopyrazol-1-yl | 90 | 4-bromopyrazol-1-yl |
| 91 | 5-bromopyrazol-1-yl | 92 | 4,5-dibromopyrazol-1-yl | 93 | 3,4,5-tribromopyrazol-1-yl |
| 94 | 4-sulfanylpyrazol-1-yl | 95 | 4,5-bis(sulfanyl)pyrazol-1-yl | 96 | 5-sulfanylpyrazol-1-yl |
| 97 | 5-iodopyrazol-1-yl | 98 | 4-iodopyrazol-1-yl | 99 | 3-iodopyrazol-1-yl |
| 100 | 3,4-diiodopyrazol-1-yl | 101 | 3,4,5-triiodopyrazol-1-yl | 102 | 3,4,5-trifluoropyrazol-1-yl |
| 103 | 3-fluoropyrazol-1-yl | 104 | 3,4-difluoropyrazol-1-yl | 105 | 4-fluoropyrazol-1-yl |
| 106 | 5-fluoropyrazol-1-yl | 107 | 3-aminopyrazol-1-yl | 108 | 4-aminopyrazol-1-yl |
| 109 | 5-aminopyrazol-1-yl | 110 | 5-methylpyrazol-1-yl | 111 | 5-ethylpyrazol-1-yl |
| 112 | 4-methylpyrazol-1-yl | 113 | 4,5-dimethylpyrazol-1-yl | 114 | 5-(sulfanylmethyl)pyrazol-1-yl |
| 115 | 4-sulfanyl-5-(sulfanylmethyl)pyrazol-1-yl | 116 | 5-aminosulfanyl-4-sulfanyl-pyrazol-1-yl | 117 | 4,5-bis(aminosulfanyl)pyrazol-1-yl |
| 118 | 4,5-bis(aminosulfanyl)-3-sulfanyl-pyrazol-3-yl | 119 | 5-ethyl-4-methyl-pyrazol-1-yl | 120 | Phenyl |
| 121 | 4-pyridyl | 122 | 3-pyridyl | 123 | 2-pyridyl |
| 124 | 1,2-dihydropyridazin-3-yl | 125 | 3,6-dihydropyridazin-4-yl | 126 | pyrimidin-4-yl |
| 127 | 1,3,5-triazin-2-yl | 128 | pyrimidin-2-yl | 129 | pyrazin-2-yl |
| 130 | cyclohexyl | 131 | 2-fluorocyclohexyl | 132 | 3-fluorocyclohexyl |
| 133 | 4-fluorocyclohexyl | 134 | 1-piperidyl | 135 | hexahydropyridazin-1-yl |
| 136 | Piperazin-1-yl | 137 | 1,2,4-triazinan-1-yl | 138 | 3,4-difluorocyclopentyl |
| 139 | 3-fluorocyclopentyl | 140 | 4-(3-chlorocyclopenta-2,4-dien-1-yl)phenyl | 141 | 4-(3,4-dichlorocyclopenta-2,4-dien-1-yl)phenyl |
| 142 | 4-(3-fluorocyclopenta-2,4-dien-1-yl)phenyl | 143 | 4-(3,4-difluorocyclopenta-2,4-dien-1-yl)phenyl | 144 | 4-(3-chloro-4-fluoro-cyclopenta-2,4-dien-1-yl)phenyl |
| 145 | 4-(3-fluorocyclopenta-2,4-dien-1-yl)phenyl | 146 | 4-(3-fluoro-4-methylcyclopenta-2,4-dien-1-yl)phenyl | 147 | 4-(3,4-difluoro-2-methyl-cyclopenta-2,4-dien-1-yl)phenyl |
| 148 | 4-(3-chloro-4-methylcyclopenta-2,4-dien-1-yl)phenyl | 149 | 4-(4-chloro-2-methylcyclopenta-2,4-dien-1-yl)phenyl | 150 | 4-(3,4-dichloro-2,5-dimethyl-cyclopenta-2,4-dien-1-yl)phenyl |
| 151 | 4-(5-methyl-3-furyl)phenyl | 152 | 4-(3-furyl)phenyl | 153 | 4-(2-furyl)phenyl |
| 154 | 4-(3-sulfanylcyclopenta-2,4-dien-1-yl)phenyl | 155 | 4-[3,4-bis(sulfanyl)cyclopenta-2,4-dien-1-yl]phenyl | 156 | 4-(3-methyl-4-sulfanyl-cyclopenta-2,4-dien-1-yl)phenyl |
| 157 | 4-(3-methyl-2-sulfanyl-cyclopenta-2,4-dien-1-yl)phenyl | 158 | 4-[3,4-bis(sulfanyl)cyclopenta-2,4-dien-1-yl]phenyl | 159 | 4-(2-sulfanylcyclopenta-2,4-dien-1-yl)phenyl |
| 160 | 4-(1-thienyl)phenyl | 161 | 4-pyrrol-1-ylphenyl | 162 | 4-imidazol-1-ylphenyl |
| 163 | 4-(1H-imidazol-2-yl)phenyl | 164 | 4-oxazol-2-ylphenyl | 165 | 4-(4-methylimidazol-1-yl)phenyl |
| 166 | Adamantyl | 167 | Fluoro | 168 | phosphanyl |
| 169 | Iodo | 170 | diiodo | 171 | 3-fluorophenyl |
| 172 | 3,4-difluorophenyl | 173 | 3,5-difluorophenyl | 174 | 4-bromo-3-fluoro-phenyl |
| 175 | 2-fluorophenyl | 176 | 4-chloro-2,6-difluoro-pheny | 177 | 2,6-difluorophenyl |
| 178 | 2,3,6-trifluorophenyl | 179 | 2,3,5,6-tetrafluorophenyl | 180 | 2,3,4,5,6-pentafluorophenyl |
| 181 | 2-chlorophenyl | 182 | 3-chlorophenyl | 183 | 4-chlorophenyl |
| 184 | 5-chloro-2-methyl-phenyl | 185 | 5-chloro-2,3-dimethyl-phenyl | 186 | 3,4-dichlorophenyl |
| 187 | 3,4,5-trichlorophenyl | 188 | 3-chloro-4,5-difluoro-phenyl | 189 | 3-chloro-4-fluoro-phenyl |
| 190 | 3-bromophenyl | 191 | 3,4-dibromophenyl | 192 | 3,4,5-tribromophenyl |
| 193 | 2-furyl | 194 | 3-furyl | 195 | 3-thienyl |
| 196 | 6-methyl-3-pyridyl | 197 | Pyrimidin-5-yl | 198 | cyclopropyl |
| 199 | cycloprop-2-en-1-yl | 200 | 2-fluorocycloprop-2-en-1-yl | 201 | 2-chlorocycloprop-2-en-1-yl |
| 202 | 2-bromocycloprop-2-en-1-yl | 203 | 2-iodocycloprop-2-en-1-yl | 204 | 2,3-difluorocycloprop-2-en-1-yl |
| 205 | 2-chloro-3-fluoro-cycloprop-2-en-1-yl | 206 | 2-bromo-3-fluoro-cycloprop-2-en-1-yl | 207 | 2-fluoro-3-iodo-cycloprop-2-en-1-yl |
| 208 | 2,3-bis(sulfanyl)cycloprop-2-en-1-yl | 209 | 2-iodo-3-sulfanyl-cycloprop-2-en-1-yl | 210 | 2-sulfanylcycloprop-2-en-1-yl |
| 211 | cyclopentyl | 212 | Cyclopenta-2,4-dien-1-yl | 213 | Cyclopenten-1-yl |
| 214 | 3,4-difluorocyclopenten-1-yl | 215 | methyl | 216 | fluoromethyl |
| 217 | difluoromethyl | 218 | trifluoromethyl | 219 | chloromethyl |
| 220 | dichloromethyl | 221 | trichloromethyl | 222 | bromomethyl |
| 223 | dibromomethyl | 224 | tribromomethyl | 225 | Vinyl |
| 226 | (Z)-2-fluorovinyl | 227 | 2,2-difluorovinyl | 228 | (E)-2-chloro-2-fluoro-vinyl |
| 229 | (E)-2-chlorovinyl | 230 | 2,2-dichlorovinyl | 231 | (E)-2-bromo-2-chloro-vinyl |
| 232 | 2,2-dibromovinyl | 233 | 1-piperidyl | 234 | morpholino |
| 235 | 4-methyl-1-piperidyl | 236 | 4-fluorol-1-piperidyl | 237 | 4,4-difluorol-1-piperidyl |
| 238 | 4-(trifluoromethyl)-1-piperidyl | 239 | 4-(trifluoromethyl)piperazin-1-yl | 240 | 4-methylpiperazin-1-yl |
| 241 | 3-methylpiperazin-1-yl | 242 | 4-sulfamoylphenyl | 243 | benzenesulfonyl |
| 244 | (1E,3Z)-2,3,4-trifluorobuta-1,3-dienyl | 245 | (1E)-2,3,4,4-tetrafluorobuta-1,3-dienyl | 246 | (E)-2,3,3,3-tetrafluoroprop-1-enyl |
| 247 | 2,2-difluorovinyl | 248 | 1,2,4-oxadiazol-3-yl | 249 | 5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl |
| 250 | 2-sulfanylacetyl)amino | 251 | carboxy | 252 | sulfanylmethyl |

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| https://lh4.googleusercontent.com/xgjEsojo1h1AN6rZQAicosuZHJu-LdYGNPo5auo8sxyl-EDfWOEqPgOlWyomhKjQOAuWTMaxipy8RHc_rw_IKDQ8KkuQqgbDB26OBXzh7PmELtmsUhfPTr6bCtYuwx63BY5__n6KAYTLuAG3F5sXMw | https://lh5.googleusercontent.com/nhSfxPYcn0zVtylxYbZulo_Lorg-w4gVWcy6kKoBl_CUA0KgCWYqBZX0_FZSCV7zvXte5j0rv2P98-zE6gv59g-LczQy-BMCdlUcc5ETzbTTO_AC8ehBsVEeOXNGtn9ETzk2lJHg-_VhdQxfN0yG0Q |
| 243-242 : Ki = 50 pM | 126-242 : Ki = 70 pM |
| https://lh3.googleusercontent.com/9AwqT17G76e0DuS6ef_1XjCRWFQXKS_IM44chcEJ5wiWDh1ZlB0_KkvfuPi1e313O8o1BcI5ljYPasaGxTcA-BlORE1Cc1J-_YhyrzyWXbuxulwhhazrO6F__FntUnaf2ut-G1wdmvOyR8Hj0ClxCg | https://lh4.googleusercontent.com/6rw-nLv2q1j6QgVuBkVoEnhZPf9DlVzyRztyGCo6bpy_AsKkDJZa-fxRZmqshjnxrZOzEDfV4e0hgwRgtl2BHZKnGxy28TNtMvXaJUQxDzPYLkuorF0GXzxUgnS3v1mneepruZ5w6V4_Ia4N5D7IKA |
| 126-158 : Ki = 90 pM | 17- 48 : Ki = 130 pM |
| **Figure 7. The best AHD Analogs with scaffold of APN, the name is a concatenation** | |

**Pharmacokinetic Profile of Novel AHD Analogs**

The properties related to ADME such as octanol-water partitioning coefficient, aqueous solubility, blood-brain partition coefficient, Caco-2 cell permeability, serum protein binding, number of likely metabolic reactions, and another eighteen descriptors related to absorption, distribution, metabolism, and excretion (ADME) were calculated by the QikProp program [46] for the new best AHD analogs (Table 7). This program is based on the method of Jorgensen []. Experimental data from more than 710 compounds including about 500 drugs and related hetero-cycles were used to produce regression equations correlating experimental and computed descriptors resulting in an accurate prediction of pharmacokinetic properties of molecules. Drug likeness (#stars) - the number of property descriptors that fall outside the range of optimal values deter-mined for 95% of known drugs out of 24 selected descript-tors computed by the QikProp, was used as an additional ADME-related compound selection criterion. The values for the predicted best active designed AHDs are compared to those computed for current anticancers targeting APN, displaying favorable pharmacokinetic profile with low number of stars indicating that the computed descriptors do not fall outside the range of 95% of known drugs.

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| --- | --- |
| https://lh6.googleusercontent.com/zzrlD8J1IKhQMZ1ZUOnyzDPXWMvuBeOBYKx5L2cCCwm1GB7hVyFiCd9D8GAE5O2ncogTlRFgGoMRMAcRKSkkVFVHXWIg_l2yt6cT8tdZvPGHeM4A9plMpCziy-5fcJLQuRMiAoZ6Xh35XjW-E4dbSw | https://lh5.googleusercontent.com/VS45mzCDyf367-PNlCGwux4Fj5SI0iR-gJS7Rn9t0X00zopzZ-uXOqZMMzSa4q7ZD_YGsfZfE5vHTKzOeBRwI3T5UHZTVSwfTdY6QXjUNTTz6whC_xRFXhAUZCsonf-KxIVJK55V3Hl8_uoLplA5bA  https://lh3.googleusercontent.com/3XjVM2-w1gULQFkZVPWwmFwhKk7cQHWNoHqhYZrbBvbbWGwooh3Za23ccmpFFNBanNcw84snHw09ePjkniKUXAIc7DYI0HAepwxtWc9VClJmlQZNfZj0mseEwTBHPbGp9HK1i-0IIYEmNVPzeAwNqw |
| https://lh3.googleusercontent.com/cF3MfYh8ZSLmLNo12U5GLJpOKHybAeTiQeZKPcfmS9LqJQgspZ2nyZz08QZkDTT8F4iNZa3HPUzQkgcbJJPwmeSX852LXsMWyY3NQh1s9KI1XAMr6qM5RASd9I53yUX3Aoa7iSijQF2ZTNfocesqXA | |
| **Figure 8**. (A, Top left) Close up of virtual hit 243-242, the most active designed AHD analog (Kipre = 50 pM) at the active site of APN. (B Top right up) Mapping of the AHD 243-242to APN inhibition pharmacophore. (C Top right down) 2D schematic interaction diagram of the best active designed AHD analog 243-242at the active site of APN. (D Bottom) Surface of the active site of APN with bound best active designed AHD analog. The binding site surface is colored according to residue **hydrophobicity: red = hydrophobic, blue = hydrophilic, and white = intermediate** | |

|  |
| --- |
| https://lh4.googleusercontent.com/OYumTHi5c6YgV6__snIgdEzd3W5Dr9BVuyQr9IIYwP58gsKAEGPjwTxGMtEcTbePOwG9wEQEdl49VQ9C9tBQcJRcjDQF2FBwEgq5zgtT3Gm7lAEiJoljfrHyXYHHY36F-j2ju9zcypWEb8ZZVvjg2A |
| https://lh6.googleusercontent.com/EkjpJ2cMW90RL5ilK8O2t-lTfSndHIF8XquafDA_6t02WUClwSBEqIhPSzKDRHGll5VJcoyYyawuTLx9a3Jgmx_LEnv0SzNRJzDp3a6z3SUUY_0hlZO3D9p6un9GrVLWvQWiVyZRrF0d4CTh9h2vcw |
| **Figure 9**. Histograms of frequency of occurrence of individual R-groups in the 95 best selected analogs mapping to four features of the PH4 pharmacophore hypothesis Hypo1 |

**Table 6**. GFE and their components for the top scoring 95 virtual AHD analogs. The analog numbering concatenates the index of each substituent R1 to R2 with the substituent numbers taken from Table 5

| N° | Analogs | MW a | ∆∆HMM b | ∆∆Gsol c | ∆∆TSvib d | ∆∆Gcom e | **Kipre  f** |
| --- | --- | --- | --- | --- | --- | --- | --- |
| - | AHD1 | 493 | 0 | 0 | 0 | 0 | 4500g |
| **1** | 1-151 | 458 | 4.64 | -2.41 | 2.22 | 0.01 | 5120 |
| **2** | 1-156 | 459 | 6.62 | -1.17 | 5.00 | 0.45 | 6200 |
| **3** | 1-159 | 459 | 6.41 | -1.37 | 4.14 | 0.89 | 7520 |
| **4** | 1-46 | 449 | 5.06 | 2.34 | 2.36 | 5.04 | 46100 |
| **5** | 1-73 | 433 | 3.92 | 3.15 | 1.86 | 5.22 | 49930 |
| **6** | 2-151 | 459 | 2.35 | -1.68 | 4.44 | -3.77 | 980 |
| **7** | 2-156 | 473 | 7.20 | -1.06 | 4.28 | 1.86 | 11480 |
| **8** | 2-73 | 447 | -0.49 | 6.01 | 3.19 | 2.33 | 14100 |
| **9** | 2-115 | 445 | 9.07 | -0.95 | 6.25 | 1.88 | 11570 |
| **10** | 3-18 | 415 | 14.08 | 0.89 | 5.73 | 9.24 | 289720 |
| **11** | 3-115 | 448 | 8.95 | 1.56 | 4.30 | 6.20 | 76720 |
| **12** | 4-151 | 460 | 3.17 | 2.17 | 3.22 | 2.12 | 12890 |
| **13** | 8-151 | 479 | 1.10 | -1.51 | 0.64 | -1.06 | 3210 |
| **14** | 8-115 | 465 | 15.16 | -1.63 | 1.81 | 11.72 | 855630 |
| **15** | 9-151 | 479 | 3.45 | -0.36 | 0.75 | 2.34 | 14180 |
| **16** | 9-154 | 493 | 3.25 | -3.79 | 2.60 | -3.14 | 1290 |
| **17** | 12-18 | 492 | 12.70 | 1.09 | 5.23 | 8.57 | 215720 |
| **18** | 17-151 | 446 | 13.96 | 1.31 | 6.60 | 8.67 | 225400 |
| **29** | 17-159 | 460 | 3.57 | -2.69 | 8.67 | -7.79 | 170 |
| **20** | 17-164 | 430 | 7.06 | 1.84 | 5.73 | 3.17 | 20400 |
| **21** | 17-48 | 439 | -0.42 | -1.71 | 6.35 | -8.48 | 130 |
| **22** | 17-49 | 439 | 8.42 | 0.88 | 5.42 | 3.87 | 27700 |
| **23** | 17-78 | 423 | 7.12 | -1.66 | 7.86 | -2.40 | 1780 |
| **24** | 26-164 | 481 | 6.42 | 0.20 | 5.07 | 1.55 | 10020 |
| **25** | 31-163 | 465 | 2.48 | -1.45 | 5.26 | -4.22 | 800 |
| **26** | 35-57 | 475 | 4.76 | 3.94 | 4.96 | 3.75 | 26220 |
| **27** | 59-151 | 492 | 4.08 | 0.84 | 1.33 | 3.59 | 24440 |
| **28** | 60-151 | 492 | 4.73 | -4.26 | 0.52 | -0.05 | 4990 |
| **29** | 60-163 | 475 | -6.58 | 7.55 | 3.65 | -2.68 | 1580 |
| **30** | 62-250 | 483 | 3.89 | -3.02 | -0.26 | 1.13 | 8330 |
| **31** | 63-18 | 461 | 7.36 | -1.48 | 1.35 | 4.52 | 36840 |
| **32** | 64-250 | 439 | 4.05 | -2.89 | 0.52 | 0.64 | 6750 |
| **33** | 69-159 | 484 | 0.72 | 0.90 | 3.83 | -2.22 | 1930 |
| **34** | 69-57 | 484 | 0.34 | 5.73 | 6.97 | -0.91 | 3430 |
| **35** | 69-250 | 400 | -2.31 | 7.63 | 1.27 | 4.05 | 29910 |
| **36** | 82-159 | 489 | -0.15 | -0.80 | 3.70 | -4.65 | 670 |
| **37** | 82-151 | 475 | 10.95 | -1.37 | 1.84 | 7.74 | 150130 |
| **38** | 84-151 | 395 | 17.43 | 2.03 | 2.33 | 17.12 | 9099450 |
| **39** | 84-159 | 409 | 19.06 | -2.13 | 5.84 | 11.08 | 648460 |
| **40** | 85-157 | 467 | 5.96 | -2.59 | 3.00 | 0.36 | 5970 |
| **41** | 88-155 | 499 | 2.40 | -0.95 | 1.71 | -0.26 | 4550 |
| **42** | 88-159 | 467 | 6.78 | -1.21 | 2.26 | 3.31 | 21630 |
| **43** | 88-115 | 439 | 10.25 | -1.87 | 0.07 | 8.32 | 193500 |
| **44** | 102-115 | 485 | 10.73 | -1.73 | -1.99 | 10.99 | 621240 |
| **45** | 102-151 | 472 | 6.45 | -4.00 | -0.25 | 2.70 | 16560 |
| **46** | 102-157 | 486 | 6.21 | -1.90 | 0.20 | 4.11 | 30790 |
| **47** | 103-157 | 450 | 8.26 | -3.21 | 3.79 | 1.26 | 8840 |
| **48** | 103-158 | 483 | 7.81 | -7.74 | 0.86 | -0.79 | 3600 |
| **49** | 104-151 | 454 | 7.54 | -4.74 | 1.09 | 1.71 | 10750 |
| **50** | 104-242 | 451 | 5.84 | 0.25 | 2.37 | 3.71 | 25840 |
| **51** | 105-159 | 450 | 8.05 | -4.02 | 3.33 | 0.70 | 6900 |
| **52** | 106-57 | 413 | 5.80 | -0.40 | 2.58 | 2.82 | 17450 |
| **53** | 106-74 | 425 | 6.76 | -3.43 | 10.65 | -7.32 | 210 |
| **54** | 106-158 | 483 | 3.75 | -3.97 | 0.55 | -0.77 | 3640 |
| **55** | 106-151 | 436 | 5.27 | -1.59 | 2.14 | 1.54 | 9980 |
| **56** | 106-242 | 433 | 3.96 | -1.44 | 2.35 | 0.17 | 5500 |
| **57** | 120-164 | 413 | 3.88 | -2.91 | 2.88 | -1.92 | 2200 |
| **58** | 122-78 | 407 | -3.96 | -0.17 | 4.04 | -8.18 | 140 |
| **59** | 123-155 | 476 | 2.87 | -7.48 | 3.43 | -8.04 | 150 |
| **60** | 126-158 | 477 | 2.31 | -9.36 | 2.10 | -9.16 | 90 |
| **61** | 126-242 | 427 | -14.40 | 6.60 | 2.16 | -9.96 | 70 |
| **62** | 130-45 | 439 | 11.51 | 1.65 | 11.83 | 1.33 | 9100 |
| **63** | 130-46 | 439 | 10.45 | 6.78 | 11.55 | 5.68 | 60960 |
| **64** | 130-57 | 412 | 5.80 | 0.70 | 8.83 | -2.33 | 1840 |
| **65** | 139-49 | 432 | 9.64 | -0.63 | 7.62 | 1.39 | 9360 |
| **66** | 122-158 | 476 | 2.87 | -7.48 | 3.43 | -8.04 | 150 |
| **67** | 139-58 | 415 | 10.63 | 0.09 | 8.19 | 2.52 | 15340 |
| **68** | 139-242 | 435 | 6.50 | 0.20 | 4.61 | 2.09 | 12680 |
| **69** | 182-157 | 477 | 5.58 | -3.57 | 2.16 | -0.15 | 4780 |
| **70** | 182-242 | 460 | 2.18 | -0.81 | 1.77 | -0.41 | 4260 |
| **71** | 127-18 | 384 | 3.85 | 4.58 | 4.56 | 3.87 | 27670 |
| **72** | 179-116 | 487 | 1.12 | -1.57 | 0.74 | -1.19 | 3030 |
| **73** | 138-58 | 433 | 9.57 | 3.04 | 7.68 | 4.93 | 44010 |
| **74** | 127-77 | 409 | 3.92 | 4.36 | 2.40 | 5.88 | 66770 |
| **75** | 193-151 | 418 | 7.41 | -3.23 | 2.75 | 1.44 | 9570 |
| **76** | 194-57 | 395 | -10.88 | 9.17 | 2.04 | -3.75 | 990 |
| **77** | 217-78 | 380 | 15.52 | -12.98 | 3.63 | -1.09 | 3160 |
| **78** | 245-78 | 454 | 6.91 | -2.03 | -0.16 | 5.04 | 46130 |
| **79** | 246-78 | 442 | 13.07 | -9.52 | -0.09 | 3.64 | 25050 |
| **80** | 204-28 | 443 | 18.78 | 2.47 | 4.64 | 16.61 | 7273880 |
| **81** | 214-57 | 431 | 3.91 | 2.92 | 3.76 | 3.08 | 19550 |
| **82** | 59-115 | 478 | -4.99 | 1.30 | -1.06 | -2.63 | 1620 |
| **83** | 85-151 | 453 | 4.91 | -4.23 | 0.68 | 0.00 | 5100 |
| **84** | 121-242 | 426 | 4.45 | 1.36 | 2.27 | 3.54 | 23970 |
| **85** | 121-45 | 434 | 1.30 | -0.55 | 4.65 | -3.89 | 930 |
| **86** | 122-250 | 360 | 1.88 | 3.14 | 4.32 | 0.70 | 6920 |
| **87** | 122-57 | 406 | 8.46 | 1.47 | 3.24 | 6.68 | 94600 |
| **88** | 123-151 | 429 | 4.60 | -3.03 | 1.96 | -0.39 | 4300 |
| **89** | 125-250 | 363 | 1.55 | 4.32 | 3.40 | 2.46 | 14960 |
| **90** | 183-151 | 463 | 5.64 | -4.32 | 0.62 | 0.69 | 6900 |
| **91** | 189-46 | 486 | 9.09 | -1.04 | 2.29 | 5.75 | 63020 |
| **92** | 189-48 | 475 | 4.63 | -1.32 | 1.72 | 1.59 | 10210 |
| **93** | 189-77 | 459 | 3.95 | -0.37 | 0.91 | 2.67 | 16380 |
| **94** | 251-151 | 396 | 9.35 | -2.12 | 1.52 | 5.71 | 61910 |
| **95** | 243-242 | 490 | -16.32 | 6.03 | 0.49 | -10.78 | 50 |

***a*** *Mw is molar mass of inhibitor;* ***b*** *∆∆HMM is the relative enthalpic contribution to the GFE change of the APN-AHD complex formation ∆∆Gcom (for details see footnote pf Table 2);* ***c*** *∆∆Gsol is the relative solvation GFE contribution to ∆∆Gcom;* ***d*** *∆∆TSvib is the relative (vibrational) entropic contribution to ∆∆Gcom;* ***e*** *∆∆Gcom is the relative Gibbs free energy change related to the enzyme–inhibitor APN-AHD complex formation ∆∆Gcom ≡ ∆∆HMM + ∆∆Gsol − ∆∆TSvib;* ***f*** *Kipre is the predicted inhibition potency towards APN calculated from ∆∆Gcom using correlation Equation B, Table 3;* ***g*** *Kiexp [39] is given for the reference inhibitor AHD1 instead of the Kipre.*

**DISCUSSION**

The most comprehensive metrics of APN inhibition by hydroxamic acid containing AHDs reported by J. Lee *et al.* [39]. Intermolecular interactions of **AHD1** and *h*APN including hydrophobic stacking interactions and hydrogen bonds were the key determinants for better affinity with the target. The exploration of the chemical AHD subspace implemented in a diverse virtual library with AHDs active conformation yielded the best R1 and R2 substituents to be accommodated by the hydrophobic pockets or rooted in other ways such as hydrogen bonds and van der Waals contacts. The strategy was executed over three orders of magnitude of experimental Ki, i.e. three pKi units taking benefit from the reported SAR continuity [39] making feasible activity prediction according similarity-property principle (SPP).

The compound 6f, N-(2-(Hydroxyamino)-2-oxo-1-(3′-fluoro-[1,1′-biphenyl]-4-yl)ethyl)-4-(methylsulfonamido) benzamide has been designed by J. Lee *et al*. with the purpose to improve both potency and solubility through removal of two fluorine atoms to keep only one compared to AHD1 (Ki = 4.5 ± 0.8 nM), they reached a potency Ki = 0.66 ± 0.06 nM []. Our AHD analogs potency prediction model computed ∆∆Gcom = - 2 kcal/mol and a potency Ki = 2.1 nM using correlation Equation B, Tables 3 and 6, presenting 6f as twice more potent than AHD1 and keeping in this way the same trend as experimental values according to which, 6f is 6-fold more potent than AHD1 regardless experimental uncertainties. The computed solubility of some AHD analogs (Table 7) is of the same order as of 6f.

The predicted most potent analogs 243-242 (50 pM) with benzenesulfonyl (243) in R1 and 4-sulfamoylphenyl (242) in R2, 126-242 (70 pM) bearing pyrimidin-4-yl in R1, 126-158 (90 pM) with 4-[3,4-bis(sulfanyl)cyclopenta-2,4-dien-1-yl]phenyl (158) in R2 keep the filling of S1 bringing better interactions and fill better the large S1’ hydrophobic pocket resulting in better affinity as displayed in Figure 10 comparing the Van der Waals interaction energy (EVDW) breakdown to APN active site residues of the best active TS AHD1 and predicted best active novel AHD analogs. This substantial stabilization will undergo medicinal chemistry verification through synthesis and biological evaluation.

**Limitations of the study**

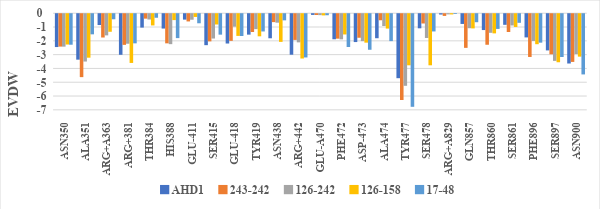
**CONCLUSION**

Structural investigation of the SAR of hydroxamic as inhibitor of new cancer target, human M1 aminopeptidase (APN) from the crystal structure of APN: AHD complex guided us during preparation of a reliable complexation QSAR model of APN activation which correlated computed relative Gibbs free energies upon complex formation with observed APN activation potencies. In addition we have derived a 3D-QSAR PH4 pharmacophore model from AHD active conformation using a training set of 37 and validation set of 9 AHDs with known activation activities. Careful analysis of interactions between the APN’s active site residues and APNs directed us in the design of an initial diversity virtual combinatorial library of new AHD analogs with multiple substitutions of hydrophobic groups in R1 and R2. A library screened by matching of the analogs to the PH4 pharmacophore permitted selection of a library subset of AHDs. This subset of 95 best virtual hits was submitted to computation of predicted activation potencies by the complexation QSAR model. The hit analogs reached predicted activities in the picomolar concentration range. The hit designed AHD analogs 243-242 (50 pM), 126-242 (70 pM), 126-158 (90 pM) and 17-48 (130pM) are recommended for synthesis and subsequent activity evaluation in APN activation assays and may lead to a discovery of novel hydroxamic potent partial APN agonists.

**Table 7**. ADME-related properties of the best designed analogs and known anticancer agents either in clinical use or currently undergoing clinical testing computed by QikProp [47]

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **AHD  a** | **#stars  b** | **MW c** | **Smol d** | **Smolhfo e** | **Vmol        f** | **#rotB  g** | **HBdon h** | **HBacc i** | **LogPo/w j** | **LogSwat k** | **LogKhsa    l** | **LogB/B  m** | **BIPcaco   n** | **#metab   o** | **Kipre      p** | **HOA   q** | **%HOA   r** |
| 243-242 | **2** | 489 | 750.8 | 10.2 | 1343.9 | 9 | 4.2 | 14.4 | -1 | -3.822 | -1.114 | -3.753 | 3.1 | 1 | **50** | 2 | **29.6** |
| 126-242 | **1** | 427 | 695 | 10.7 | 1222.4 | 8 | 4.2 | 12.9 | -1 | -3.239 | -1.078 | -3.263 | 5.8 | 2 | **70** | 2 | **34.8** |
| 126-158 | **0** | 476 | 780.1 | 30.4 | 1391.8 | 8 | 3.8 | 9.4 | 2.9 | -5.645 | -0.212 | -1.544 | 119.4 | 5 | **90** | 3 | **80.9** |
| 17-48 | **0** | 439 | 738.3 | 43.2 | 1312.0 | 10 | 5 | 9.9 | 0.8 | -3.264 | -0.601 | -1.934 | 9.1 | 6 | **130** | 2 | **49.1** |
| 122-78 | **0** | 407 | 693.8 | 10.1 | 1218.8 | 8 | 3 | 9.9 | 1.3 | -3.967 | -0.644 | -1.914 | 63.1 | 4 | **140** | 3 | **66.8** |
| 17-159 | **1** | 459 | 781.2 | 54.9 | 1408.1 | 9 | 5 | 7.4 | 2.7 | -4.730 | 0.005 | -1.447 | 24.8 | 7 | **150** | 2 | **67.5** |
| 106-74 | **1** | 423 | 730.2 | 234.4 | 1314.9 | 7 | 4 | 10.4 | 0.4 | -3.641 | -0.485 | -2.930 | 10.7 | 3 | **170** | 2 | **47.8** |
| 82-159 | **1** | 488 | 784.5 | 32.1 | 1423.7 | 8 | 3 | 8.4 | 3.5 | -6.314 | 0.081 | -1.554 | 144.2 | 3 | **210** | 3 | **86.1** |
| 31-163 | **1** | 465 | 774.2 | 38.3 | 1375.5 | 8 | 4.2 | 8.4 | 2.8 | -5.694 | -0.120 | -1.786 | 88.9 | 4 | **800** | 3 | **78.5** |
| **AHD1** | **0** | 493 | 771.2 | 92.2 | 1362.4 | 8 | 3.2 | 10.4 | 1.8 | -5.410 | -0.425 | -2.187 | 36.0 | 1 | **4500** | 3 | **65.7** |
| 6f | **0** | 457 | 755.2 | 92.2 | 1332.5 | 8 | 3.2 | 10.4 | 1.4 | -4.720 | -0.489 | -2.360 | 36.0 | 1 | **660** | 3 | **63.2** |
| Phebestin | **0** | 441 | 767.3 | 226.8 | 1402.3 | 13 | 3.5 | 7.2 | 0.8 | -3.527 | -0.479 | -1.639 | 8.6 | 7 |  | 2 | **35.3** |
| Tosedostat | **0** | 406 | 730.2 | 372.6 | 1313.8 | 11 | 2.2 | 8.6 | 1.4 | -3.066 | -0.637 | -2.086 | 67.3 | 4 |  | 3 | **67.9** |
| Bestatin | **0** | 308 | 592.7 | 242.2 | 1035.2 | 10 | 3.2 | 5.4 | -0.247 | -1.877 | -0.624 | -1.179 | 13.8 | 5 |  | 2 | **45.9** |
| Probestin | **0** | 491 | 829.4 | 465.3 | 1573.1 | 15 | 5 | 11.9 | -0.862 | -0.929 | -0.651 | -1.411 | 0.6 | 8 |  | 1 | **5.6** |
| Amastatin | **4** | 474 | 798.1 | 509.9 | 1476.1 | 16 | 4.5 | 10.7 | -1.614 | -1.464 | -1.524 | -3.115 | 0.1 | 7 |  | 1 | **0** |

*a designed AHD analogs and known antituberculotic agents, Table 6; b drug likeness, number of property descriptors (24 out of the full list of 46 descriptors of QikProp, ver. 3.7, release 14) that fall outside of the range of values for 95% of known drugs; c molar mass in [g.mol-1] (range for 95% of drugs: 300–500 g.mol-1 ) [22]; d total solvent-accessible molecular surface, in [Å2] (probe radius 1.4 Å) (range for 95% of drugs: 300–1000 Å2); e hydrophobic portion of the solvent-accessible molecular surface, in [Å2] (probe radius 1.4 Å) (range for 95% of drugs: 0–750 Å2); f total volume of molecule enclosed by solvent-accessible molecular surface, in [Å3] (probe radius 1.4 Å) (range for 95% of drugs: 500–2000 Å3); g number of non-trivial (not CX3), non-hindered (not alkene, amide, small ring) rotatable bonds (range for 95% of drugs: 0–15); h estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can assume non-integer values (range for 95% of drugs: 0.0–6.0); i estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can assume non-integer values (range for 95% of drugs: 2.0–20.0); j logarithm of partitioning coefficient between n-octanol and water phases (range for 95% of drugs: -2 to 6.5); k logarithm of predicted aqueous solubility, logS in [mol.dm–3] is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid (range for 95% of drugs: -6.0 to 0.5); l logarithm of predicted binding constant to human serum albumin (range for 95% of drugs: -1.5 to 1.5); m logarithm of predicted brain/blood partition coefficient (range for 95% of drugs: -3.0 to 1.2); n predicted apparent Caco-2 cell membrane permeability in Boehringer-Ingelheim scale in [nm s-1] (range for 95% of drugs: < 25 poor, > 500 nm s -1 great); o number of likely metabolic reactions (range for 95% of drugs: 1–8); p predicted inhibition constants Kipre. Kipre in pM was predicted from computed ∆∆Gcom using the regression Equation B shown in Table 3; q human oral absorption (1 = low, 2 = medium, 3 = high); r percentage of human oral absorption in gastrointestinal tract (80% = high); \* star in any column indicates that the property descriptor value of the compound falls outside the range of values for 95% of known drugs*



**Fig. 10**. Van der Waals component of Molecular Mechanics intermolecular interaction energy (Eint) breakdown to residue contributions in EVDW [kcal.mol-1]: of the most active AHD1 and the best analogs

**ABBREVIATIONS**

2D   Two-dimensional

3D    Three-dimensional

ADME   Absorption, distribution, metabolism, and excretion

AHD     Hydroxamic acids inhibitors

AHDx   Training set of Hydroxamic acids

VAHDx   Validation set of Hydroxamic acids

∆∆Gcom    Relative complexation GFE

GFE    Gibbs free energy

∆∆Gsol   Relative solvation GFE

HBA     Hydrogen bond Acceptor

HBD    Hydrogen bond Donor

HMM     Enthalpy component of GFE

HOA    Human oral absorption

HYD   Hydrophobic

HYDA   Hydrophobic Aliphatic

Ki   inhibitory concentration

M1 metalloaminopeptidase 1

MM   Molecular mechanics

MM-PB   Molecular mechanics–Poisson–Boltzmann

PDB   Protein Data Bank

APN Aminopeptidase N

PfA-M1 *Plasmodium falciparum* M1 aminopeptidase

PH4 Pharmacophore

QSAR Quantitative structure–activity relationships

RMSD Root-mean square deviation

SAR Structure–activity relationships

TS Training set

VS Validation set

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