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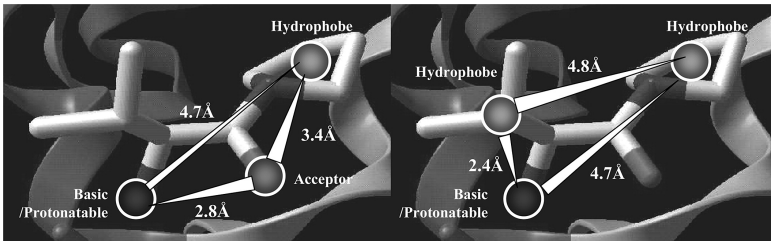

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Structure-Based Virtual Screening for Low Molecular Weight Chemical Starting Points for Dipeptidyl Peptidase IV Inhibitors

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Structure-based virtual screening was performed against the target dipeptidyl peptidase IV (DPP-IV) to identify good chemical starting points for medicinal chemistry. A database of available compounds was filtered by calculated physical properties and undesired chemistry. This database was matched against two in-house designed DPP-IV pharmacophores, and the hits from these pharmacophore searches were docked into a DPP-IV crystal structure. Compounds were then selected for testing and 51 active compounds were identified from a list of 4000 compounds tested. These had activities ranging from 30% to 82% when tested at a concentration of 30 μM in an enzyme inhibition assay.

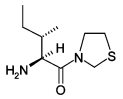
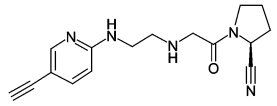
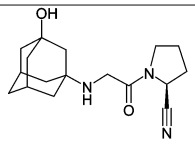
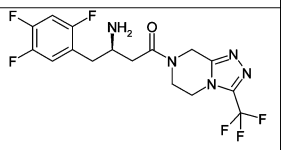
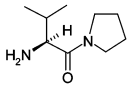
Introduction

There is significant interest in the identification of inhibitors of dipeptidyl peptidase IV (DPP-IV) for the treatment of Type 2 diabetes.[1] A major role of DPP-IV is the degradation of the peptidic hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)[2]. These incretin hormones are involved in the physiological control of insulin release and the regulation of blood glucose. Inhibition of DPP-IV in normal and diabetic rats has been shown to prevent the degradation of GLP-1, leading to enhanced insulin secretion and improved glucose tolerance.[3,4] DPP-IV is able to selectively cleave Xaa-Pro and Xaa-Ala dipeptides from the N-terminus of peptides and proteins (where 'Xaa' is any amino acid).

A variety of DPP-IV inhibitors have been patented and published in recent years.[5] Some earlier reports identified cyanopyrolidine-containing inhibitors, which target the Serine-630 residue in the S1 pocket of DPP-IV. There are also a variety of reversible DPP-IV inhibitors which have been reported. A selection of these are shown in Table 1.

Several DPP-IV crystal structures have recently been published, which allows structure-based drug design to be used in the search for new and different classes of DPP-IV inhibitors. Published DPP-IV crystal structures show a variety of bound ligands including Val-Pyr (**5**) in the 1N1M[11] structure. Other published structures include 1PGQ,[12] 1NU6, and 1NU8,[13] and they have very similar conformations around the active site, adding confidence that this may be a suitable target for virtual screening. Knowledge of a protein−ligand complex opens up the possibilities for computational tools to be used with greater confidence to identify new chemical classes of inhibitors. In particular, ligand-based methods such as pharmacophore design[14] and subsequent pharmacophore searching, 3D similarity searching,[15] and shape-based searching[16] can be used to complement protein−ligand docking.[17]

Table 1. Selection of Current DPP-IV Inhibitors

Compound	Reference	IC_{50} (DPP-IV)	K_i (DPP-IV)	Compound Structure
1	Probiodrug/ Merck[6]	-	130nM	
2	Novartis[7]	-	11nM	
3	Novartis[8]	3.5nM	-	
4	Merck[9]	18nM	-	
5	Val-Pyr[10]		255nM	

The identification of chemically tractable, low molecular weight DPP-IV inhibitors as starting points for drug discovery is therefore of great interest. A high throughput screen (HTS) was previously run in-house on around 800 000 compounds with a view of identifying a suitable starting point for the development of an orally active, reversible DPP-IV inhibitor. This HTS showed an extremely low hit rate, with the vast majority of actives turning out to be irreversible inhibitors. Fewer than 100 compounds showed better than 30% inhibition at 10 μM in the HTS. This gave a hit rate for the HTS of around 0.012% for actives with 30% inhibition at 10 μM. None of these compounds was judged as suitable

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for follow-up due, in most cases, to their irreversible mechanism of action, while the remainder were considered to have poor synthetic tractability.

Virtual screening, defined as the use of computational methods to identify a subset of compounds for testing, is being increasingly used to complement traditional HTS approaches.[18] This can be particularly valuable when the screening technique of choice has a low throughput. In addition, it can also be useful to highlight less potent compounds, but where the interactions with the receptor can be inferred from the virtual screen, so that these compounds may provide better start-points for further optimization. There are many publications detailing a range of protocols for virtual screening including database pre-filtering,[19] docking protocols,[20] and postprocessing of results.[21]

Virtual screening was therefore deployed against DPP-IV to attempt to identify low molecular weight starting points that may have been missed by the HTS. It is likely that these have a relatively low potency, or that they belong to a more sparsely populated region of chemical space where the statistics of large-scale biological screening and the preferences of medicinal chemists may have made them harder to identify. It was therefore decided to test a smaller number of compounds (around 4000 in total) at a higher compound concentration of 30 μM (compared with 10 μM in the HTS), using virtual screening methodology to select the compounds for testing. It was hoped that this process may identify lower molecular weight starting points which can be developed by medicinal chemistry.

Given the availability of a number of crystal structures with bound ligands for DPP-IV, one obvious strategy would have been to dock the database of compounds directly into the active site. In this case, however, there were a number of compelling reasons to choose a pharmacophore-based strategy as an initial filter in the virtual screen. First, there is considerable literature[11,12,13] and in-house evidence that several specific interactions are extremely favorable (notably between a ligand basic group and two glutamic acid residues on DPP-IV). It may be possible to identify completely different binding modes, but those that make use of these interactions were considered to be more likely to be active. Second, any agreement between the pharmacophore-based alignment and the pose predicted by the docking increases confidence in the results. Finally, while it is possible to dock 800 000 compounds using commercially available docking algorithms, the practicalities and logistics remain daunting. It was therefore decided to use a DPP-IV crystal structure to build a pharmacophoric description of the ligands and to screen the database of compounds against this as a prefilter. The hits from the pharmacophore would then be docked into the DPP-IV crystal structure and these compounds would then be ranked and a further selection made. Finally, compounds could be visually inspected and a final list of compounds prepared for testing.

2. Results and Discussion

A subset of 800 000 compounds which could be made available for screening was used for the starting point for the virtual screen. Filtering on physical properties and chemical filters reduced this number to around 500 000 compounds. Tautomer, protonation state, and stereogenic center enumeration using the in-house software and Corina[23] increased the number of structures to around 750 000 in the single conformer database. The number of structures in the multiconformer database is substantially higher as up to 500 conformations from each compound are built. This multiconformational database was then run against the two designed DPP-IV pharmacophores. Twenty thousand hits from each of the pharmacophores were selected based on the RMSD overlap of the compound with the pharmacophore and the overlap with the excluded volume of the active site. A single-conformer database of the 40 000 selected molecules was then docked into the DPP-IV crystal structure using GLIDE.[22] Ten docked poses for each compound were postprocessed and the top 8000 compounds were picked. Clustering and then visual inspection was used to select a final 4000 compounds for screening (Figure 3).

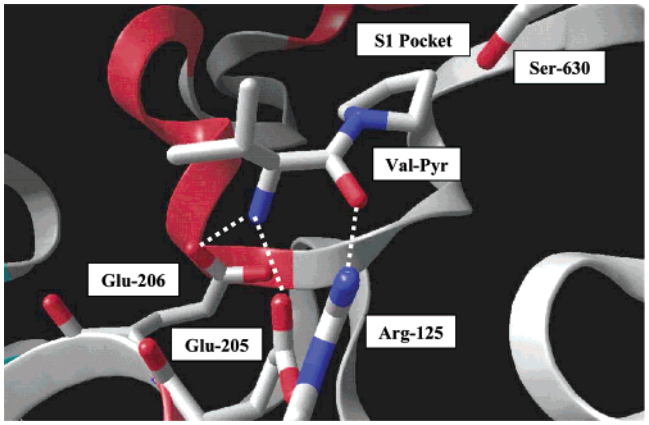

Figure 1. DPP-IV structure with Val-Pyr bound showing hydrogen bond interactions with protein.

The activity cutoff for the assay based on the data was set so that any compound with at least 30% inhibition at 30 μM was defined as an active. Compounds between 25% and 30% inhibition at 30 μM were designated as weakly active and the remainder inactive. According to these criteria, 51 active compounds were identified and 11 weakly active compounds. The hit rate for the actives can be calculated as 1.28% in this virtual screen. This is not particularly high, but when compared with the hit rate of the HTS of 0.012% it shows that it is a substantial improvement against random screening. But it is important to point out that the hit rate of the two assays cannot be compared fairly, as the HTS was run at 10 μM compound concentration so it could have missed many of these weaker actives in the HTS 'noise'. The use in this project of virtual screening to rescue an unproductive HTS meant that it was not necessary to rerun the entire compound collection at a higher concentration. Virtual screening identified more compounds to follow-up than the HTS. Clustering of the actives using a tanimoto distance of 0.2 and Daylight fingerprints[23] showed 25 singleton actives. Eight cluster seeds were also found which had between one and three nearneighbors at tanimoto 0.2.

Unfortunately, no genuinely potent compounds were identified in the virtual screen. It was hoped that some more potent compounds may have been identified which,

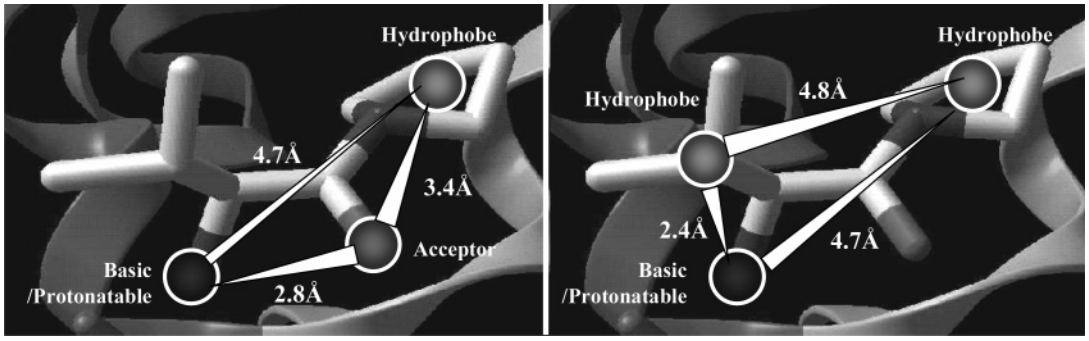

Figure 2. The three-point pharmacophores used for DPP-IV virtual screen (left is pharmacophore 1; right is pharmacophore 2).

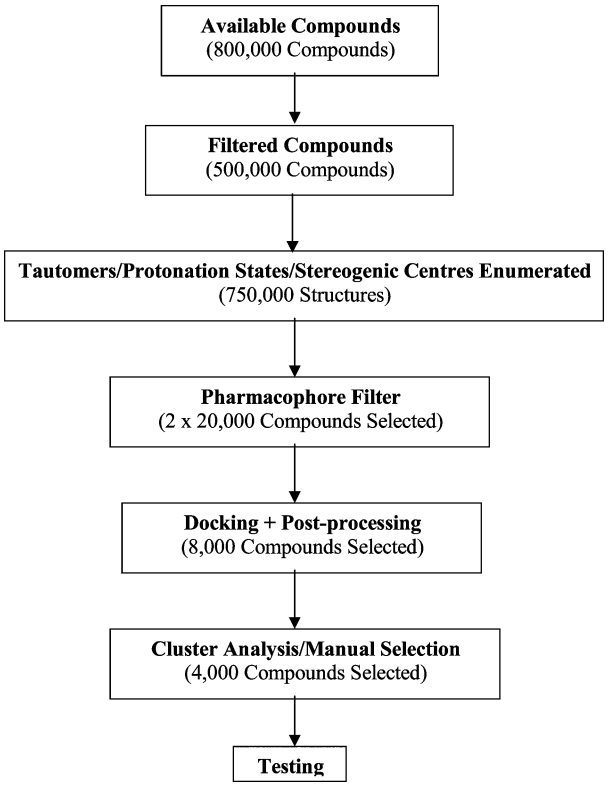

Figure 3. Virtual screening protocol.

possibly due to them being in more sparsely populated clusters, could have been missed by the HTS. This was not the case, and the most potent example had a % inhibition of 81.9% at 30 μM.

Figure 4 shows a selection of the cluster seeds from the virtual screening hits which had at least one near-neighbor using a tanimoto distance of 0.2. The docked structures of these cluster seeds were analyzed to rationalize the SAR and allows ideas for how these templates may be expanded. There was also a selection of interesting singletons from the virtual screen which had no active near-neighbors. A selection of these are shown in Figure 5 with the active data in brackets. None of these compounds (or clusters of related compounds) were identified as active from the HTS so these starting points would not have been identified from a medicinal chemistry campaign based around the HTS output. It is also important to note that many of these examples can be considered to be novel and different to most published DPP-IV inhibitors.

The docked binding mode of these compounds was also analyzed to look for regions of the active site which

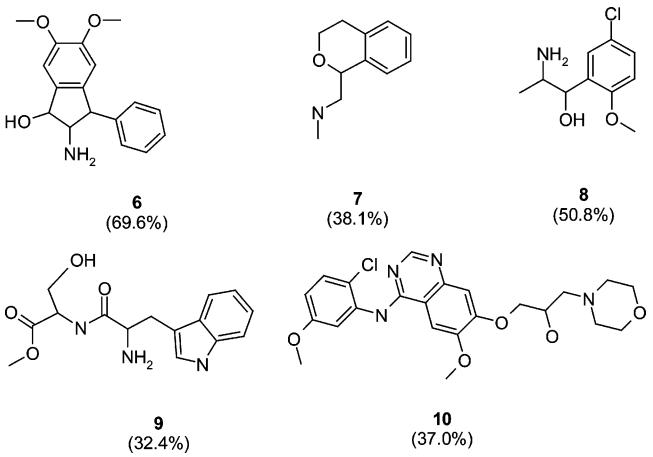

Figure 4. Cluster seeds of a selection of virtual screening hits (bracketed figure denotes % inhibition at 30 μM of cluster seed).

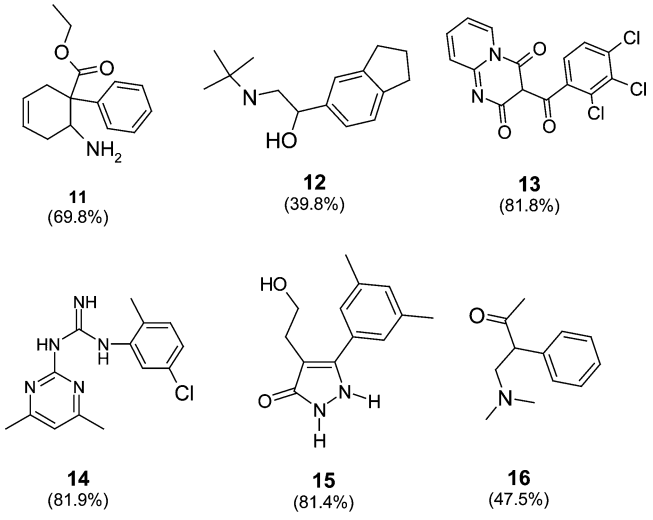

Figure 5. Selection of active singletons from virtual screening (bracketed figure denotes % inhibition at 30 μM of compound).

could be explored further using these templates. Note that compound **13** does not have a basic group or a hydrogen bond donor as drawn, but the enumerated enol tautomeric states do provide potential hydrogen bond donors. A further set of weakly binding actives were also identified but none of these were analyzed further as it was felt that they were not potent enough to be of interest.

The performance of the two designed pharmacophores was also retrospectively analyzed. Pharmacophore 1 found 29 302 matches within the searched database and pharmacophore 2 found 41 102 matches. Out of the 51

active compounds, 28 came from pharmacophore 1 and the remaining 23 were found from pharmacophore 2. This gives a hit rate for the confirmed actives of 0.095% from pharmacophore 1 and 0.057% from pharmacophore 2. These hit rates are higher than the HTS hit rate of 0.012% but again the difference in compound concentration should be considered as increasing the compound concentration in an HTS to 30 μM would increase the hit rate. However, this would also increase the noise and the number of false positives in the HTS. It is also important to point out that these hit rates are calculated on the confirmed actives and without further testing it is not known what additional actives the pharmacophores if used in isolation may have identified. This analysis shows that pharmacophore 1 was more successful in finding a greater number of the confirmed hits and with a better success rate than pharmacophore 2. To further assess the performance of each stage of the virtual screen the number of the identified actives which would have been found by simply taking the top hits from each pharmacophore (based on the RMSD overlap and excluded volume) was investigated. The results show that none of the identified actives would have been found, although without further testing it is not known if other interesting actives would have been found instead. The extra value of the docking step is that it gives further validation to the defined pharmacophore, and the docked structure can then be used to rationalize the SAR and generate ideas for expansion. This shows that the docking protocol was an important additional step at the end of the virtual screen, but this cannot be further categorized without more experimental testing.

3. Conclusions

The work shows how virtual screening techniques can be used to identify new and novel potential starting points for chemistry when conventional HTS runs have not yielded any useful hits. It was felt that running two pharmacophoric searches effectively utilized the knowledge of the DPP-IV crystal structures and also of known DPP-IV active compounds. The three-point pharmacophores were run, as they effectively exploited the structural data but also produced novelty in the identified hits. Four-point pharmacophores found very few matches, and the identified compounds were very similar to the Val-Pyr starting structure. The docking protocol was also selected to improve the hit rate of the compounds selected for testing by giving a more effective scoring function than simply the RMSD overlap against the pharmacophore and the excluded volume overlap with the active site. The docking protocol also gives confidence in the predicted pharmacophoric binding mode which can then be used to help follow-up the compounds. The constructed pharmacophores were then used to postprocess the docked poses before finally scoring the compounds for selection. It would be interesting to compare the hit rate of this virtual screen against a protocol which simply docked all of the available compounds into a DPP-IV crystal structure, but this would require further computational and experimental work. The compounds identified here are generally of a low molecular weight and therefore of a fairly low potency but the virtual screening has identified possible chemical starting points that were not identified from the HTS.

Initially, these compounds were followed up by the testing of near-neighbors and related compounds in the compound collection and then analyzing the data for further SAR. This information was then used by medicinal chemists to assess the synthetic feasibility of initiating a chemistry campaign based around these chemical starting points. Chemical feasibility was the major consideration at this point and whether there was enough chemical scope for modification to enable the substantial increase in potency, which would be required from these relatively weak hits. No formal cutoff in potency was used to assess these actives. As an HTS had already been completed, it was expected that the hits would be relatively weak, so chemical feasibility and novelty were the two major issues. Some of the larger compounds were only considered if the activity was reasonable, but again, there was no formal cutoff for this, and due to the relatively small numbers of actives, using a ligand efficiency measure was not required for compound prioritisation. Obtaining an X-ray crystal structure of the compounds of interest bound in DPP-IV along with molecular modeling would also assist in following up these chemical starting points. With the continually increasing number of DPP-IV structures being released, extra interactions between the bound DPP-IV inhibitors and the protein can also be identified to drive an increase in potency.

4. Experimental Section

4.1. Database Preparation. The database of available compounds for testing was available in a SMILES format.[24] The database was initially prefiltered, based using a cutoff of <35 heavy atoms, ClogP between -2 and 4, and using additional chemical filters developed in-house in order to remove functional groups unattractive to medicinal chemistry. A tight cutoff was used on molecular weight and ClogP to try and ensure that the compounds would make reasonable chemical starting points. The remaining SMILES in the database were processed with the in-house ionization and tautomer model using the Leatherface molecular editing program.[20] This rule-based model corrects states that are unlikely to exist under normal physiological conditions and enumerates the most relevant tautomeric forms. 3D structures were then generated from SMILES using Corina,[25] which was also used to enumerate all stereoisomers. The MMFF94[26] force field was then used to optimize Corina structures in the database to improve geometries. For the pharmacophore searching, a multiconformer database was built with a maximum of 500 conformers for each structure using OMEGA,[27] with the settings ewindow = 6 and rms = 0.6 and the torsional parameters tuned in-house to improve the conformation of some of the rotamer groups. The multiconformer database was then screened against a single conformation pharmacophore using in-house software. For the compounds selected from the pharmacophore search for docking, the input conformation was taken as the MMFF-minimized Corina structure.

4.2. Pharmacophore Building. The DPP-IV crystal structure 1N1M[11] was used to build two pharmacophores for the virtual screen which had the inhibitor Val-Pyr bound in the active site (Figure 1). The presence of these pharmacophores was searched in the 3D multiconformer database of available compounds. The crystal structure clearly shows some important interactions, most noticeably a hydrophobic group in the S1 pocket and an interaction between the basic amine and two glutamate residues (Glu-205 and Glu-206) in the active site.

It is important to note that the majority of reported DPP-IV inhibitors have a hydrophobic group which could potentially fill the S1 pocket and a protonatable group (usually a basic amine) which can interact with Glu-205 and Glu-206. This is especially apparent in the reversible DPP-IV inhibitors in the

literature (e.g. compounds **1**, **4**, and **5**). The hydrophobic definition used also covered a range of five- and six-membered ring systems, some which although not particularly hydrophobic would be a suitable size to be accommodated in the S1 pocket. The basic pharmacophore point was also extended to allow a range of defined hydrogen bond donors. These should interact well with the Glu residues and may allow greater novelty in the identified hits. There are also a further two interactions shown in the DPP-IV crystal structure. The hydrogen bonding interaction of the carbonyl group in the ligand with Arg-125 and a hydrophobic interaction from the isopropyl region of the ligand. It was therefore decided to build two three-point pharmacophores. Both of these had the hydrophobic S1 pocket group and a basic/protonatable group included, but one had the described hydrogen bond acceptor and the other had the additional hydrophobic group (Figure 2). Two three-point pharmacophores were run instead of a single four-point pharmacophore to increase the diversity of the matched compounds. A four-point pharmacophore will identify a smaller number of more chemically similar compounds so it was decided to run with two three-point pharmacophores.

4.3. Pharmacophore Searching. An in-house pharmacophore search method[20] was chosen to search the multiconformer database against the two pharmacophores described above. This program allows the pharmacophore to be described in terms of SMARTS[24] definitions for each pharmacophoric point. A range of functional groups and motifs are coded into each of the pharmacophore definitions as SMARTS and used in the search. Clique detection is used to match appropriate groups in each compound with the pharmacophore and align those that match onto the query. It is also possible to allow the active site of the protein to be considered as an excluded volume in the search, and this allows hits to be ranked on the overlap with the pharmacophore as well as the potential clash of the overlayed ligand with the active site. It is routine to search a database of around 1 million compounds (with potentially around 150 million conformers) in under 1 h on a 50 node Linux farm with this software.

4.4. Preparation of DPP-IV Protein. The crystal structure of DPP-IV with PDB code 1N1M[11] was used as the basis of the docking experiments. All crystallographic water molecules were removed, and hydrogen atoms were added to the protein and ligand in Maestro from Schrödinger[28] using the predicted protonation states of the amino acid residues at pH 7.4. The crystal structure was then minimized in Macromodel[29] with the heavy atoms fixed to allow the hydrogens to be positioned more optimally. The MMFF force field with a distance dependent dielectric constant of 4 was used, and 500 iterations of conjugate gradient optimization were run.

4.5. Docking Protocol. The prepared crystal structure was used for the docking campaign with the Val-Pyr ligand used to define the active site with a box size of 12.5 Å. GLIDE[22] was used for the docking calculation since it was successful in reproducing DPP-IV crystal structure binding modes in preliminary experiments and has regularly been shown in publications and in-house to be one of the most reliable docking algorithms.[31,32] The hits from the pharmacophore search were input into GLIDE as an sdf file using the preparation procedure described in section 2.1. GLIDE was used with default settings, and the top 10 docked poses for each docked structure were retained. Note that at this stage there may be more than one structure of each compound to be docked due to enumerated stereoisomers, and various possible tautomeric and protonation states.

4.6. Postprocessing of Docking Results and Compound Selection. The top 10 docked poses for each ligand were used for the postprocessing. The purpose of the postprocessing of docking results is to remove docked poses that may be scored well by the docking algorithm but have unlikely interactions which make the binding mode less favored. It is common in docking algorithms that the experimental binding mode is identified but it cannot be assumed that it is the top-ranked pose.[31] The postprocessing procedure is therefore designed to assist in the identification of the correct docked pose for each ligand, which in turn should improve the scoring and therefore the ranking of ligands. Initially, all of the docked poses were run through the two designed pharmacophores, and only docked poses, which passed one of the pharmacophores, were accepted. This ensures that the docked poses agree with the original binding mode identified when the compound hits the pharmacophore. Further postprocessing was then performed on these docked structures using in-house software that generates descriptors for various ligand−receptor interactions. These include measures of steric complementarity, clash with the active site, polar−lipophillic interactions, and lipophillic surface area exposed to solvent. After the docked poses have been postprocessed, the top-ranked pose (using the Glidescore) for each compound is taken and the compounds ranked on this. The selected hits were then clustered using Daylight fingerprints with a tanimoto of 0.2. Clusters which were populated with >10 near-neighbors were thinned out to ensure good diversity of the compounds to be tested. The remaining compounds were then manually inspected, and a final list was compiled for testing.

4.7. Determination of Activity of Test Compounds. The purpose of this screen is to determine activity of test compounds at a concentration of 30 μM to find novel compounds that inhibit human DPPIV activity. Using a fluorescent assay test, compounds were assessed for their ability to inhibit the DPP-IV activity in a human colonic cell line extract (Caco extract). The ability of the enzyme to cleave the synthetic substrate H-Gly-Pro-7-amino-4-methylcoumarin to produce free AMC was measured on a Fusion plate reader (excitation 360 nm, emission 465 nm).

4.8. Compound Activity Analysis. The active compounds were then analyzed by clustering using Daylight[23] fingerprints to group the compounds into related structural chemical classes. SAR for these actives can then be identified, and future modifications of the hits can be investigated. The potential of these actives as a reversible drug-like DPP-IV inhibitor can then be judged in collaboration with medicinal and synthetic chemists.

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