Current Status of Virtual Screening as Analysed by Target Class

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Abstract: *In silico* virtual screening for drug discovery has become a hot topic in medicinal chemistry research during the last 5 years, growing from a largely academic pursuit concerned principally with validating the methods used, to a major early-stage technique for lead discovery in the pharmaceutical industry. In this review we highlight a few recent successes in ligand docking associated with virtual screening, paying particular attention to four major target classes of pharmaceutical interest (G Protein-Coupled receptors, nuclear hormone receptors, kinases, proteases). We also discuss some emerging trends in the field, some common limitations, and how they are being overcome.

Key Words: Virtual screening, docking, kinase, GPCR, protease, receptors, review.

INTRODUCTION

Virtual Screening encompasses a range of overlapping technologies involving the use of computers to aid in the identification of new leads for protein or nucleic acid drug targets. As such, the measure of success of the technique differs from the traditional measure of success in medicinal chemistry – a new marketed drug – and is perhaps seen best in context of comparison with other lead-finding techniques such as analogue-based drug design, mechanism-based enzyme inhibitors, serendipity and high-throughput screening (HTS). In this review we discuss the different ways in which the major drug classes have been targeted to successfully generate new leads that may not have been discovered by conventional analogue-based drug design. The primary focus of this review is the use of high-throughput docking of large chemical databases in a *structure-based* context however it should be noted that the complementary technique of high-throughput screening of 2- and 3-dimensional structures of small molecules to match a known pharmacophore [1-4] (*analogue-based*) is also commonly referred to as virtual screening. In some areas of medicinal chemistry research, notably G Protein-Coupled Receptor (GPCR) research, pharmacophore-based methods are well entrenched due primarily to the relative maturity of the technology and the obvious relative lack of GPCR X-ray crystal structures from which to embark on structure-based drug design.

In a structure-based context, virtual screening is the process by which compounds are analysed *in silico* for shape and electronic complementarity (docking) for a receptor and then ranked/scored in order of goodness of fit. This ranking is then used to decide which compounds should be subsequently ordered or synthesised and then tested against the target of interest. In an ideal setting, the compounds chosen for testing should exhibit nanomolar to low micromolar activities, and if the scoring functions used are sufficiently accurate, the observed experimentally derived parameters such as IC_{50}, EC_{50}, K_i, K_{act} etc are hopefully in line with the docking-derived rankings. The aim is to obtain new lead molecules independent of any prior knowledge of existing leads. This technique relies for its predictive accuracy on a number of underpinning technologies such as detailed knowledge of the protein or DNA/RNA target structure, reliable computational techniques (algorithms) for conformational searching and docking low energy conformations of the flexible ligands into the targets, and finally on accurate predictions of binding affinity (scoring) in order to rank and prioritise compounds for *in vitro* testing.

The increased popularity of molecular docking and virtual screening in the past 5 years is evidenced by the rapid increase in papers published (Figure 1). Most literature in the 1980's and 1990's was concerned with the development of computational methods for reliably docking single ligands into a known X-ray crystal structure of a known protein and to best approximate the experimentally observed binding conformation and interactions (Binding pose). Since the year 2000 there has been an explosion in the number of protein structures in the PDB with a concomitant rise in the number papers describing docking and virtual screening.

Several recent comparisons of ligand-based and structure based methods [3, 5-11] have been published, along with comparisons of virtual screening and HTS [12-20]. This review also does not cover the multitude of papers over the last decade where docking of a few synthesised inhibitors is used to attempt to rationalise relative binding potencies, or the considerable ongoing effort in validating both the docking methodologies themselves and the subsequent scoring and ranking of hits.

TARGET STRUCTURE

Until comparatively recently, the major source of high (atomic) resolution structures of biomolecular targets such as proteins, DNA and RNA was X-ray crystallography, in the form of the publicly accessible PDB Database. In recent times, the complementary technique of Nuclear Magnetic Resonance (NMR) has enabled the determination of structures

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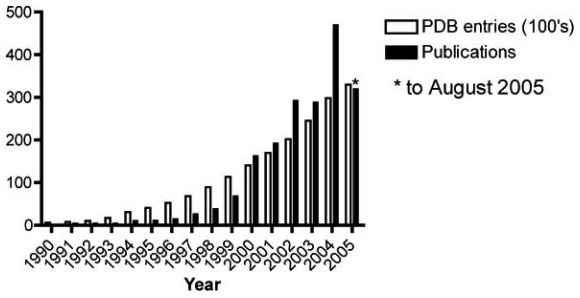


Fig. (1). Growth in Publications utilising docking and virtual screening, and the growth in numbers of publicly available crystal structures in the RCSB Protein Databank since 1990. Source: Scifinder Scholar, http://www.rcsb.org/pdb.

of proteins which had previously failed the original bottleneck of crystallisation. However, since the publication of the human genome, large numbers of potential drug targets do not currently have a publicly available structure from which to commence a structure-based drug design program. This issue has normally been addressed through the use of protein homology modelling. Homology models necessarily are of uncertain accuracy [21] and the question arises as to whether they are suitable for docking studies.

This is most dramatically the case for G-Protein Coupled Receptors (GPCRs). There is only one available structure of a GPCR in the PDB, that of bovine rhodopsin. An additional complication is that it is of the receptor in the inactive conformation. One study [22] has concluded that homology models based on the "unactivated" state of rhodopsin may be suitable for the virtual screening based discovery of antagonists, but not of agonists. They and others [23-25] have developed new "knowledge-based" and "pharmacophore-based" methods to manually create a number of predicted agonist-bound (activated) forms of the receptor which were then found to reliably extract known agonists from a seeded library.

In general, homology models are considered overall to be moderately structurally reliable if the sequence identity is >40% [26]. Other researchers using kinases (CDK2) [27] and a protease (FactorVIIa) [27] have concluded that where the overall sequence homology *in the ligand binding region* is greater than 50%, homology models suitable for reliable virtual screening can be obtained. Homology models have also been used for virtual screening of SARS Coronavirus protease [28], kinases [29], and nuclear hormone receptors [30, 31].

It is worth noting that X-ray and NMR structures themselves are not immune from errors. Recent papers [32, 33] discuss the implications of insufficient understanding of the structure calculation process from electron density as applied to virtual screening and other forms of structure based drug design.

VIRTUAL SCREENING PROGRAMS AND DATABASES

Since the introduction [34] and development [35] of the original docking program DOCK, from the Kuntz lab in 1982, a large number of other docking packages have become available from both commercial and academic groups (Table **1**). The different programs generally use an approach where the ligand is treated as flexible, and the protein rigid, although newer approaches do account in a

Table 1. Commonly used Docking Programs and Protocols

Gold	http://www.ccdc.cam.ac.uk/products/life_sciences/gold/
DOCK	http://dock.compbio.ucsf.edu/
Autodock	http://www.scripps.edu/mb/olson/doc/autodock/
FlexX	http://www.tripos.com/
Glide	http://www.schrodinger.com/
ICM	http://www.molsoft.com/products.html
Affinity	http://www.accelrys.com/
C^2.Ligandfit	http://www.accelrys.com/products/cerius2/cerius2products/c2ligandfit.html
3DPL™	http://www.chemnavigator.com/cnc/products/3dpl.asp
LIDAEUS	http://www.cyclacel.com/research_programmes/licensing.htm
PRO_LEADS	http://www.protherics.com/
Fred	http://www.eyesopen.com/products/applications/fred.html
Dockvision	http://www.dockvision.com/
Arguslab	http://www.planaria-software.com/index.htm
MOE	http://www.chemcomp.com/Corporate_Information/MOE_SBD.html
BioMedCAChe™	http://www.fujitsu.com/
Slide	http://www.bch.msu.edu/labs/kuhn/web/projects/screening.html
Surflex-Dock	http://www.biopharmics.com
Slide	http://www.bch.msu.edu/labs/kuhn/web/projects/screening.html
Ph4Dock	Tokai University School of Medicine [73]
Cdocker and Sdocker	Internal program/protocol at Eli Lilly and Company [74, 75]
Pro_Select	Internal program/protocol at Protherics [76, 77]
FFLD	http://www.biochem-caflisch.unizh.ch/ [78]
Flog	Merck Research Laboratories [79]
HierDock/HierVLS	http://www.wag.caltech.edu/home/wag/index.html [80]

limited way for protein flexibility (see limitations section later). The methods used by the docking programs to sample conformational space of the ligand usually rely on either Monte Carlo, genetic algorithm, fragment-based or molecular dynamics approaches. A detailed analysis of the different methodologies used in these differing packages is beyond the scope of this review, however a number of published comparisons of the various programs and protocols and the scoring functions they utilise have appeared recently [22, 36-72].

It is worth noting that a large number of the papers appearing in the scientific literature are in fact being published by the commercial software manufacturers themselves and in many areas these papers outweigh the numbers of academic or pharmaceutical industry papers in the field.

Enrichment factors are frequently cited as a measure of the relative success of a docking package to a particular problem. In brief, enrichment factors are a means to quantify the relative return of active compounds from a database containing a large number of decoys versus the statistical probability of discovering those hits at random. Numerous analyses [81, 82] of the relative success or otherwise of various docking packages and protocols have been reported.

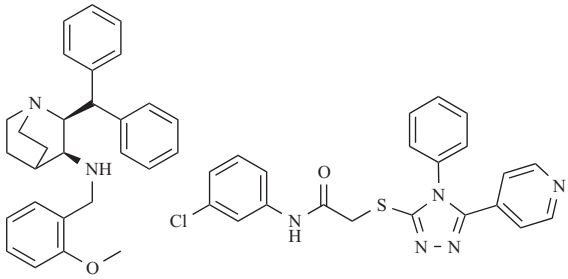
There are now a large number of vendors providing both screening libraries of compounds for testing and virtual libraries for virtual screening such as ACB-Eurochem, Ambinter, Asinex, Chembridge, ChemDiv (Chemical Diversity), ChemNavigator, ComGenex, Enamine, IBScreen, Integrity, InterChim, KeyOrganics, Leadquest, Life Chemicals, Maybridge, Nanosyn, NCI (National Cancer Institute), NCI Diversity, Otava, Peakdale, Pharmeks, PubChem, Ryan Scientific, Sigma-Aldrich, Specs, and TimTec. Of the commercial compound vendors, virtually all of these provide at least 2-dimensional databases of their compounds free that can be converted to 3-dimensional representations with tools such as CONCORD and CORINA [83, 84]. Recently the ZINC database [85] was established to provide a "ready-to-go" sets of databases for the virtual screening community. Additionally, commercial or subscription-based virtual databases of compounds such as MDL's MDDR (Medical Drug Data report), Comprehensive Medicinal Chemistry (CMC), Available Chemicals Directory (ACD), Derwent's World Drug Index, Cambridge Crystallographic Database are available. For a recent discussion of various databases and their limitations, see Olah *et al.* [86].

TARGET CLASS

As would be expected given their respective dominance of the world drug market, GPCR's (52% of world drug market) [87] and protein kinases [88] are popular targets of virtual screening. However whereas the Protein Data Bank contains 1473 kinase structures (as at 12/8/05 with 106 structures pending), GPCR-based high-throughput molecular docking depends on the reliability of homology modelling based on the one available X-ray structure, that of bovine rhodopsin, as discussed above. Other current "hot targets" are proteases [89] and nuclear hormone receptors [90-92] for which hundreds of X-ray and NMR structures are available.

G PROTEIN-COUPLED RECEPTORS

The Neurokinin receptors (NK1, NK2 and NK3) bind endogenous peptidic neurotransmitters such as neurokinin A and B and substance P, and are a molecular target for inflammation and pain research. Evers and Klebe [24, 93] have successfully utilised their MOBILE (Modeling Binding Sites Including Ligand Information Explicitly) approach to find antagonists such as **2** of the Neurokinin 1 receptor from a virtual screening screen against rhodopsin-based homology models. This is an approach where known ligands are placed into the active site of a preliminary homology model (or ensemble of models) and the new model is refined once more by optimising ligand-protein side-chain interactions. They used the known NK1 antagonist CP-96345 **1** in their protocol to ultimately produce new structurally unrelated antagonists like **2.**



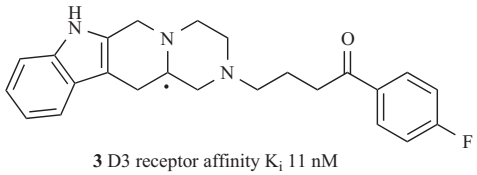
CP-96345 **1** ASN-1377642 **2**
NK1 antagonist (K_i 251 nM)

Fig. (2).

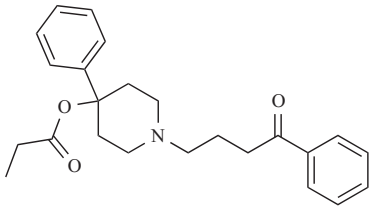
Becker *et al.* [94, 95] have reviewed the use of their proprietary (Predix Pharmaceuticals) PREDICT package to generate models of GPCR's *independent* of the rhopdopsin structure. Their software also implicitly mimics the membrane environment of the receptor. They used the software to generate a range of GPCR models and then subsequently used DOCK to screen multiple vendor databases, and claim nanomolar and micromolar hits against 5HT1a, 5HT4, NK1, D2 and CCR3 receptors, however the structures of these hits have not at the time of writing been divulged.

Varady *et al.* [96] have discovered the first potent inhibitors of a biogenic amine binding GPCR (D3) *via* a hybrid pharmacophore-docking based approach. The D3 receptor has been proposed as a target for a range of neurological conditions, including schizophrenia, Parkinson's Disease, drug abuse, and depression. Initially a homology model of the D3 receptor was constructed from the rhodopsin structure, followed by structural refinement in a simulated membrane-water environment to generate multiple binding site conformations that were clustered. An initial pharmacophore-based search of the NCI database using a pharmacophore derived from 10 known potent D3 ligands provided 6727 compounds which were subjected to a second round of structure-based screening using Cerius^2 docking into multiple representations of the binding site. These hits were augmented with 20 known D3 ligands to confirm that the docking protocol could retrieve real binders. Two rounds of scoring and structural similarity searching reduced the hit

set to 1314 compounds. 20 Compounds were tested for affinity to the D3 receptor with 11 having K_i's of between 11 nM and 2.6 μM (e.g. **3**,**4**, Fig. **3**).



3 D3 receptor affinity K_i 11 nM
stereochemistry undefined



4 D3 receptor affinity K_i 429 nM

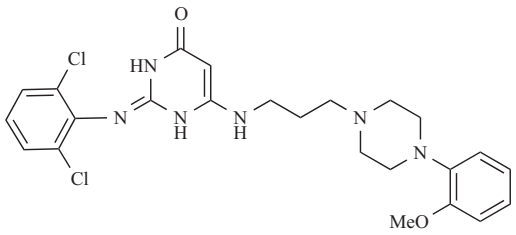
Fig. (3).

Antagonists of the alpha1A adrenergic receptor are under investigation as anti-hypertensive agents and mediators of benign prostatic hypertrophy. Virtual screening against a rhodopsin-based homology model by researchers at Aventis [97] has yielded potent compounds utilising a hybrid approach similar to that discussed above for the D3 receptor. An initial pharmacophore search using CATALYST of the Aventis corporate collection yielded a subset (~23 000 compounds) that were docked using a alpha1A receptor-validated protocol using GOLD and scored using the consensus scoring module in SYBYL . The 300 top-scoring molecules were subjected to similarity analysis in UNITY and 80 compounds were subsequently chosen for assay. Approximately half (38) of the compounds exhibited >50% inhibition of radioligand binding at 10 μM (e.g. **5**-**8**, Fig. **4**).

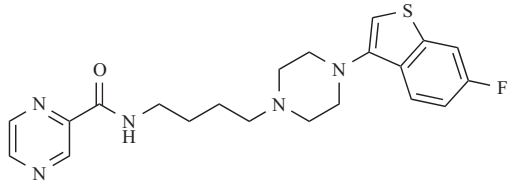
The human P2Y and P2X receptors are an extensive class of GPCR's with heavily overlapping affinities for known ligands. In an effort to dissect the physiological roles of these subclasses, selective ligands are urgently required. Using an in-house database of 500 compounds, Hiramoto *et al.* have identified new endogenous surrogate ligands for human $P2Y_1$ receptors [98] based on screening against a rhodopsin-based homology model. The ability of AUTODOCK to return known $P2Y_1$ agonists and antagonists was checked by doping the database with several known $P2Y_1$ ligands. All were returned amongst the top 30 hits. 21 of the top-ranked compounds were selected for *in vitro* functional screening. Three compounds previously unknown as $P2Y_1$ ligands were identified as agonists of the $P2Y_1$ receptor. 5-Phosphoribosyl-1-pyrophosphate (PRPP, **9**) was a moderately potent agonist (ED_{50} 15 nM) when compared with a known agonist ADP (0.14 nM). Furthermore in calcium release assays, PRPP showed good subtype selectivity, being inactive at $P2Y_2$, $P2Y_6$, and P2X2 receptor and only possessing weak activity at the $P2Y_{12}$ receptor.

KINASES

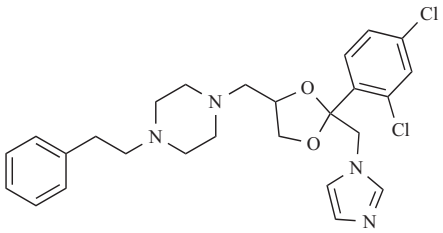
Kinases are currently a major focus of pharmaceutical design with oncological [99, 100] targets accounting for approximately 75% of all research on kinase inhibitors [101] with other work such as kinase involvement in inflammation [102] also in progress. There are estimated to be 518 kinases [103] in the human genome ("The Kinome") [104], and these constitute one of the major pharmaceutical challenges of the 21st century [88]. 5 Compounds are in clinical use (gefitinib



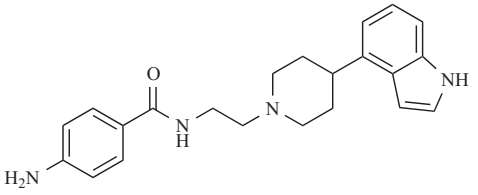
5 Alpha1A receptor affinity K_i 3.6 nM



6 Alpha1A receptor affinity K_i 1.4 nM

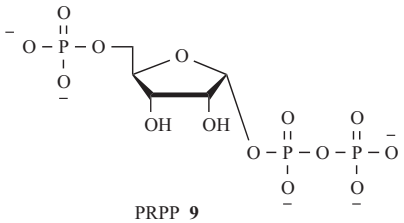


7 Alpha1A receptor affinity K_i 300 nM

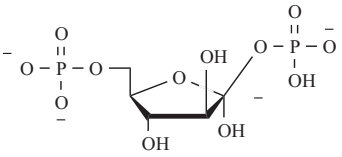


8 Alpha1A receptor affinity K_i 9.5 nM

Fig. (4).



PRPP **9**
P2Y1 agonist ED_{50} 15 nM



D-fructose 1,6-bisphosphate **10**
P2Y1 agonist ED_{50} 14 μM

Fig. (5).

(Iressa), fasudil (Fasdil), rapamycin, erlotinib (Tarceva) and imatinib (Gleevec)). At least 30 tyrosine kinases inhibitors are in clinical trials for cancer alone [105]. One of the principal challenges in kinase research is that the vast majority of compounds are targeting the ATP-binding sites of the enzymes. These active sites are highly conserved [106] although some changes are seen across classes, and this results in significant specificity issues [107-109]. In a testament to the finely-tuned nature of kinase selectivity, researchers at Johnson and Johnson have shown in a recent series of papers how a class of compounds originally investigated as ATP-site targeted CDK-1 inhibitors [110] became potent VEGFR-2 inhibitors after 2 rounds of synthesis and testing [111]. Although currently a much underutilized approach [88] other researchers approach this problem by targeting the substrate peptide-binding, SH2, SH3 and other domains, the so-called ATP non-competitive approach [108, 112-118]. In common with it's popularity in other fields of drug design, virtual screening for kinase inhibitors [119] has targeted both ATP-competitive and ATP-non-competitive approaches.

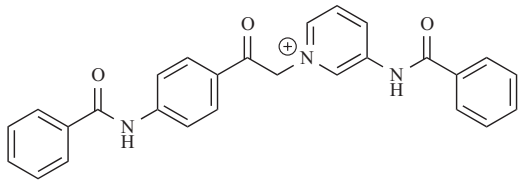
Inhibitors that Target the ATP Binding Site

The success of Gleevec (Imatinib Mesylate) **13**, for the treatment of chronic myelogenous leukemia (CML) has resulted in a number of groups targeting the same kinase (BCR-ABL tyrosine kinase). Peng *et al.* [120] have described a virtual screening program using DOCK and the ChemDiv (www.chemdiv.com) database of 200 000 compounds to find 1000 leads which were refined by similarity analysis to generate a diverse list of 15 compounds for purchase and testing. Compounds **11** and **12** were found to possess good cell-based activities (IC_{50}'s of 24 and 30 μM respectively)

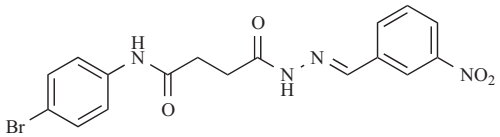
Akt (protein kinase B) is known as a major target of oncological research. Forino *et al.* [121] have obtained low micromolar potency inhibitors of Akt (protein kinase B) from a FlexX and GOLD virtual screening of the Chembridge database. Compounds **14** (IC_{50} 2.6 μM) and **15** (IC_{50} 4.5 μM) are of different structural classes but both are true ATP-competitive inhibitors of Akt.

Researchers at Bayer Germany [122] have discovered Adenosine kinase inhibitors by an undisclosed virtual screening protocol that led to hits such as the 2-aryl-ozazolopyrimidine **16**. From that point a purely traditional medicinal chemistry program led to the development of compounds such as compound **17** which upon further elaboration yielded compound **18**, a low nanomolar inhibitor (stereochemistry not disclosed).

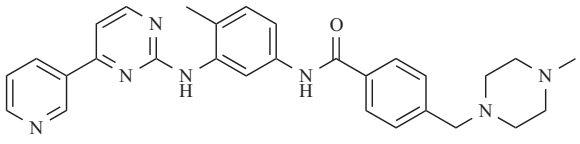
Also with a view to cancer chemotherapy, Astra-Zeneca researchers [123] have used a composite pharmacophore-virtual screening docking protocol to discover inhibitors of Checkpoint Kinase-1 (Chk-1). An initial *in silico* filter of the Astra-Zeneca in-house compound collection to provide compounds with an adenine-mimicking element as defined by a hydrogen donor and an acceptor within 1.35-2.40Å of one another, provided a "short list" of ~250 000 candidates



11 (IC_{50} 24 μM)

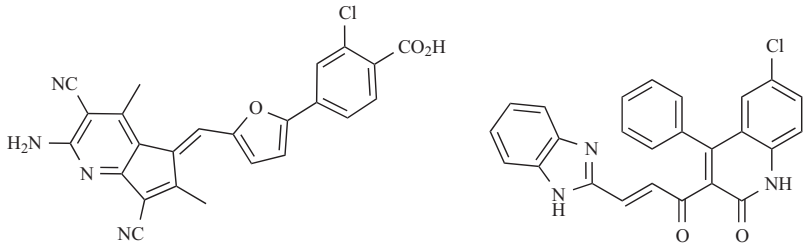


12 (IC_{50} 30 μM)



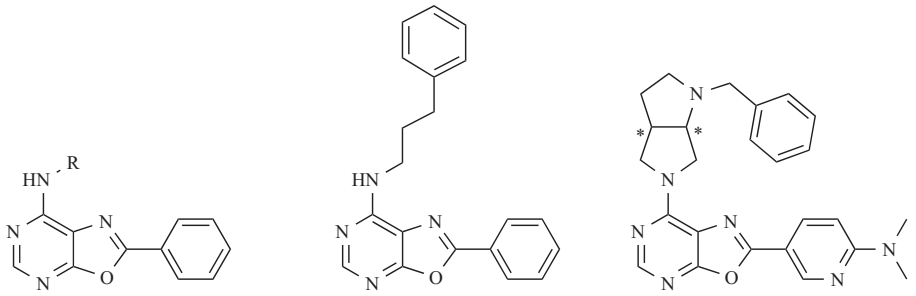
Gleevec **13**

Fig. (6).



14 **15**

Fig. (7).

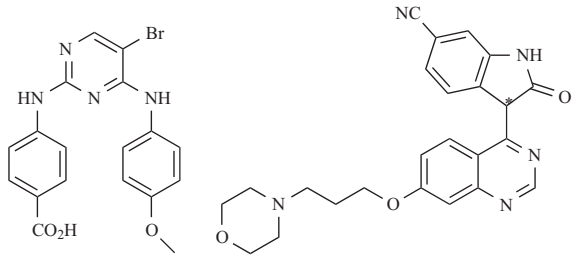


16 **17** **18**

IC_{50} 230±60 nM IC_{50} ~10 nM

(Fig. 8).

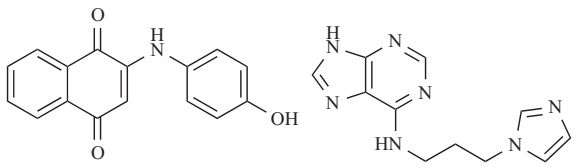
for a docking study. These were docked using a guided FlexX-Pharm protocol into the ATP-binding pocket of the Chk-1 kinase domain crystal structure. After scoring the top 250 compounds were considered by visual inspection, taking into account unrealistic conformations and unfavorable interactions with the binding site. Finally 103 compounds were selected for testing in a cell-based assay. 36 compounds possessed IC_{50}'s ranging from 110 nM to 68 μM e.g. **19**,**20** and represented 4 distinct chemical classes (chemotypes).



19 (IC_{50} 450 nM) **20** (IC_{50} 4μM) (stereochemistry not disclosed)

Fig. (9).

Toledo-Sherman *et al.* [124] from Protana Inc. have used virtual screening to target the EphB2 Tyrosine kinase. GOLD docking of a small number (95) of known ATP binding site ligands validated the method, and subsequent screening of a Lipinski-filtered [125] version of the Chemical Diversity (San Diego) database yielded 250 candidate molecules. These were then combined with a second set of molecules selected by Pharmacophore-based approaches and assayed by Frontal Affinity Chromatography interfaced to Mass Spectrometry (FAC-MS). This is a technique where the target of interest is immobilized on a solid support column and the mixture of ligands is eluted through the column with high affinity ligands being retained longer. The most potent compounds were also subsequently assayed *via* conventional ELISA techniques to determine IC_{50}'s. Compounds from several structure classes e.g. **21**,**22** were obtained with IC_{50}'s ranging from 5-200 μM.



21 (IC_{50} 38±4 μM) **22** (IC_{50} 5.2±1.5 μM)

Fig. (10).

Protein kinase CK2 (casein kinase II) is a drug target of Novartis Switzerland [126] who employed a virtual screening strategy utilizing a homology model based on a plant (*Z. mays*) structure. In common with many publications over the past few years the human crystal structure was not available at the time the work began. The Novartis corporate

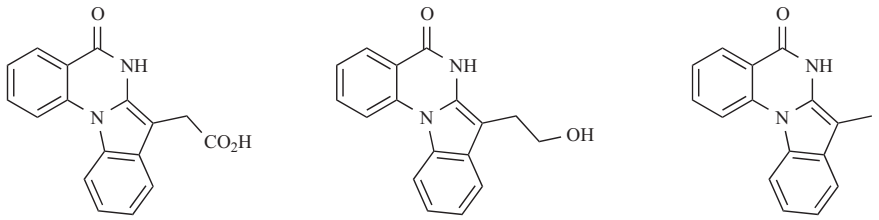
collection of 400 000 structures was searched using DOCK, with the top-ranking hits after 3 rounds of scoring-based filtering and a final filtering by visual inspection supplying 12 compounds for assay. 4 Compounds from 4 different chemotypes exhibited >50% inhibition at 10 μM in an *in vitro* assay, with Compound **23** possessing nanomolar potency and significant selectivity for CK2 over a panel of 20 other kinases. 2 Analogues of **23** were subsequently tested to examine to the importance of the carboxylate interaction with Arg43 of the enzyme, predicted to be a major determinant of selectivity.

Cyclin-Dependent Kinase (CDK) inhibitors have been found by Wu *et al.* [127] from Cyclacel using the docking program LIDAEUS. A database (unspecified) of 50 000 commercially available chemicals was screened and 148 hit compounds were selected for assay, together with 28 randomly selected decoys. Among the reported hits were 2-amino-4-heteroarylpyrimidines **26-28** with micromolar potency against CDK2 and 4, but no significant activity against PKCα and ERK-2. Investigation of the docking modes of **26-28** suggested the synthesis of **29** which possessed enhanced potency as well being reported to exhibit low micromolar (1.5 μM) anti-proliferative activity in several human tumor cell lines. These authors also crystallized all four figured inhibitors with CDK2 and examined the induced fit compared to the original crystal structure and the apo form of the enzyme to both to confirm the accuracy of the docking technique and design new compounds. The X-ray structures showed that substitution of the exocyclic aminopyrimidine nitrogen with an extra binding arm (as in **28**, **29**) appears to increase potency ten-fold by switching the binding mode, and increasing hydrophobic contacts with the enzyme.

In a subsequent paper [128] this group also describe the modification of these leads to generate more potent inhibitors based on the thiazolyl-pyrimidine scaffold. Compounds possessing moderate selectivity for CDK-2 over CDK-4 were obtained, with the best potency being in the low nanomolar range (compound **31**), and also submicromolar anti-proliferative activity was attained.

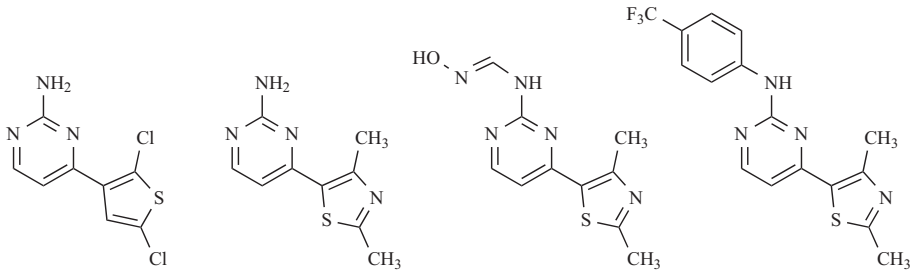
Inhibitors that Do Not Target the ATP Binding Site

Very high resolution crystal structures [129] of the tyrosine kinase p56 Lck (Lymphoid T Cell Tyrosine Kinase) have been obtained in complex with short phosphotyrosine peptides. This interaction site, the Src homology-2 domain (SH2) has been targeted by Huang *et al.* [116]. An initial screen of 2 million compounds using DOCK produced a library of 25 000 structures based on normalized van der Waals attractive energies. These compounds were subjected to a second round of docking which incorporated a more rigorous conformational search and site-based minimization to generate a subset of 1000 compounds which were subjected to a similarity search to derive 196 candidate molecules for *in vitro* screening. 17% of the selected compounds were inhibitors at 100μM, with compounds **32** and **33** being the most potent, although modest in comparison



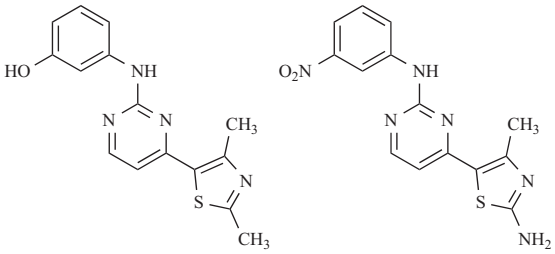
23 (IC_{50} 80 nM) **24** (48% inhibition at 10 μM) **25** (12% inhibition at 10 μM)

Fig. (11).



	26	**27**	**28**	**29**
CDK-2 IC_{50}	17 μM	13 μM	2 μM	1 μM
CDK-4 IC_{50}	3 μM	30 μM	27 μM	6 μM

Fig. (12).



	30	**31**
CDK-2 K_i	0.06 μM	0.002 μM
CDK-4 K_i	0.21 μM	0.05 μM

Fig. (13).

to Ac(pY)EEI **34** (K_D 0.1 μM). Follow-up experiments in cell-based assays confirmed their activity at ~75 and 80% inhibition at 100μM.

PROTEASES

Since the success of HIV-protease and ACE inhibitors in man, proteases are now considered as validated chemotherapeutics targets for a number of diseases, and accordingly a large number of protease inhibitors are currently under clinical investigation [89, 130]. Over 1500 X-ray crystal and NMR structures of proteases are publicly available in the PDB, together with a large but unknown number of proprietary structures in-house inside the pharmaceutical industry. This constitutes a large number of target structures for virtual screening and yet virtual screening has not been widely pursued in this field.

This may in part be due to the relatively frequent successes of conventional analogue-based and mechanism-based drug design in the area, or it may be due to the fact that proteases frequently undergo a significant "induced fit" on ligand binding [54, 131]. This is perhaps most dramatically evidenced by the large change in conformation on ligand binding of HIV-1 protease (Figure 15). Other factors that may impinge heavily on the success of protease-targeted virtual screening (and consequently the number of publications in the field) is the heavy reliance on accurate predictions of partial charges and protonation states of catalytic residues and ligands in the active sites [132-137].

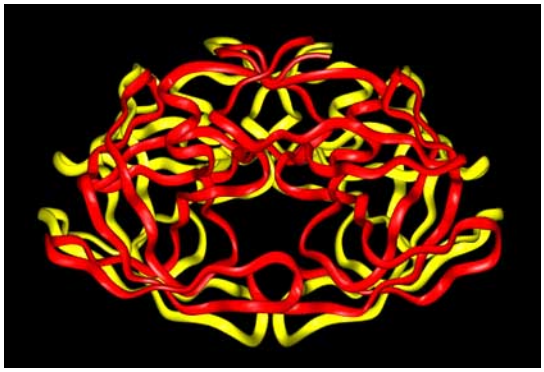


Fig. (15). Apo (yellow) and ligand-bound (red) conformations of HIV-1 protease.

Cysteine Proteases

Since the emergence of severe acute respiratory syndrome (SARS) in 2002/2003, and the rapid identification of a coronovirus as the causative agent [138,139], and it's genetic sequencing [140] the SARS Coronavirus 3CL Protease has been a principal target for the search for an anti-SARS drug. Before the crystal structure of the SARS protease [141] and the closely related human strain coronavirus strain 229E protease [142] had been determined, virtual screening had been used to identify inhibitors of the protease based on homology models derived from other human coronavirus strains. Chen *et al.* described a virtual screen of the MDL Comprehensive Medicinal Chemistry

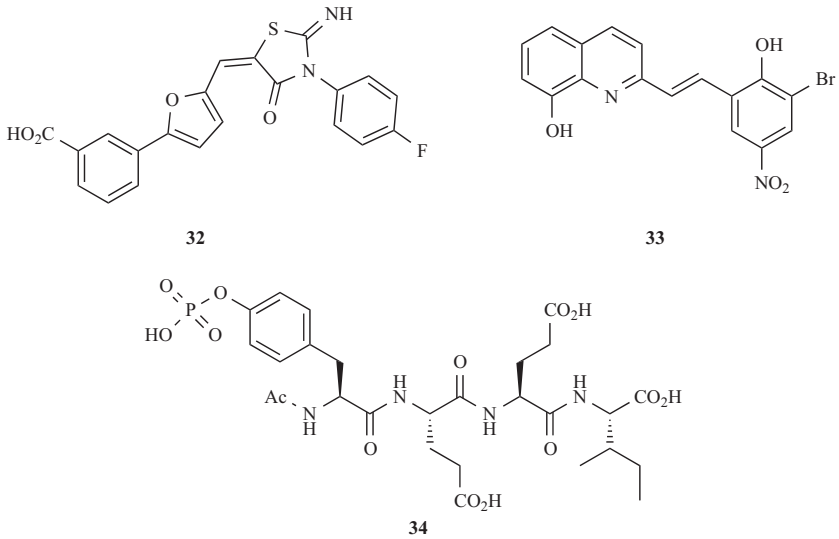
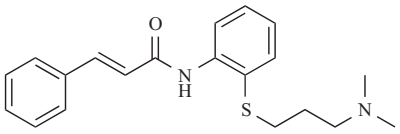


Fig. (14).

database (MDL-CMC) [143] using DOCK 4.0 and Autodock which provided Cinanserin **35** (a known 5HT receptor antagonist) as a lead compound (IC_{50} 5 μM). In this study they targeted the His-Cys catalytic dyad of the protease as the centre of the search site, and predict a contact of the active site thiol/thiolate with the cinnamoyl double bond as well as a charge-charge interaction of the dimethylamino group with Glu166 of the enzyme S1 pocket, and the active site His41 in a hydrogen bond with the amide carbonyl oxygen.



Cinanserin **35**

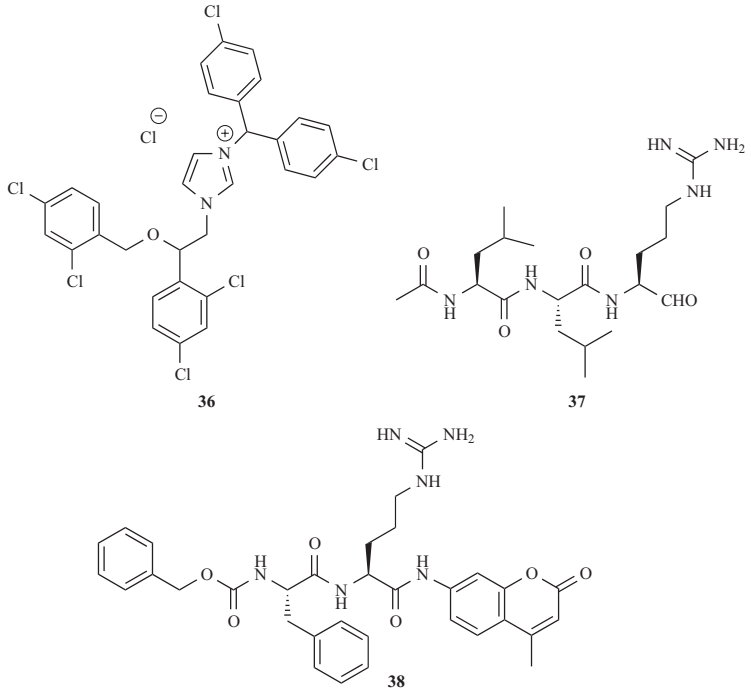
Fig. (16).

After the publication of the SARS protease crystal structure, a docking study by Rajnarayanan *et al.* [144] using FlexX and a mixture of known protease inhibitors and prefiltered reactive thiol-reactive compounds from Maybridge, Leadquest, ACD and NCI lead to a mixture of covalent and non-covalent potential inhibitors. Known HIV protease inhibitors featured prominently in the list, and a second round of suggested inhibitors was designed utilising a range of catalytic "warheads" from the covalent binding leads. Unfortunately no *in vitro* data was divulged.

Liu *et al.* [28] have reported a diverse series of inhibitors of the SARS protease derived from the NCI Diversity Set, ACD, and MDDR databases screened using DOCK. Calmidazolium chloride (**36**, K_i 61 μM), AcLeuLeuArginal (**37**, K_i 84 μM), and Z-PheArg-amc (**38**, K_i 178 μM).

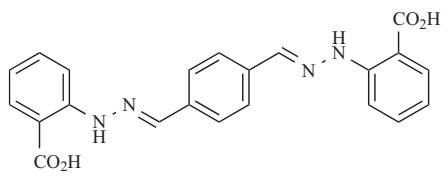
The cysteine proteases from the Trypanasoma, Leishmania and Plasmodium genera are emerging as prime targets against parasitic diseases especially in developing countries. The cysteine proteases from *Plasmodium falciparum* (falcipain-2 and –3, and cruzain from *Trypanasoma cruzii* are amongst the targets recently identified. Avery's group at Mississippi [145] have recently published the results of a virtual screen in search of non-covalent cysteine protease inhibitors of these an other parasitic targets. As a first step for accurate docking the *Plasmodium falciparum* proteases were carefully examined for the protonation state of acidic and basic residues as the protease is found in the highly acidic food vacuole. Using GOLD to dock a heavily filtered (60 000 from 241 000) subset of the Chembridge database, 84 compounds were selected for testing. 24 of these possessed activity against one or more proteases and 20 exhibited anti-plasmodial activity. Compound **39** had IC_{50}'s of 1.0 and 4.9 μM against falcipain 2 and 3 respectively, and EC_{50}'s of 35 and 41 μM against chloroquine-sensitive and resistant strains of *P. falciparum.*

Researchers at Johnson & Johnson [146] have reported the successful generation of a series of nanomolar non-covalent inhibitors of Cathepsin S based on a micromolar hit **40** from a virtual screening of the in-house J&J database (~248 000 compounds) using DOCK. Due to a number of



36 **37** **38**

Fig. (17).



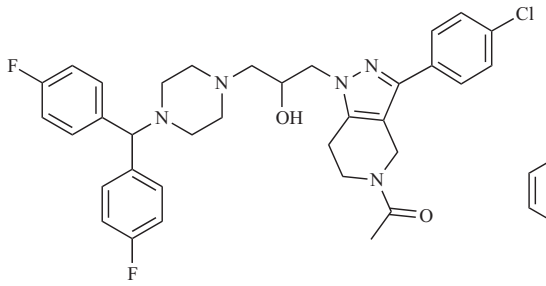
39

Fig. (18).

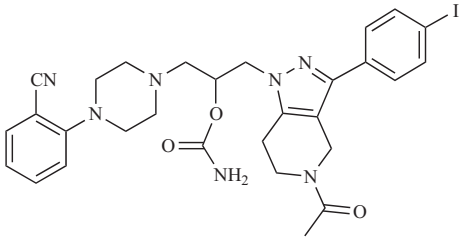
factors including cross reactivity with the Adrenergic $\alpha 1_a$, $\alpha 1_b$, and $\alpha 1_d$ receptors, compound **41** was modified to replace the aryl piperazine with a benzimidazolone derivative resulting in JNJ10329670 (**42**) [147] with somewhat lower Cathepsin S potency (IC_{50} 100 nM) but with dramatically better selectivity and 40-75% oral bioavailability [148].

Pang *et al.* [149] have obtained inhibitors of human adenovirus cysteine proteinase (hAVCP) with a new mode of action. Screening of the ACD using the docking program EUDOC suggested 30 candidate molecules of which 3 were purchased and one (TNFN, **43**) was active. TNFN had a two-step inhibition mechanism. Firstly a reversible association with K_i of 3.1 μM, followed by irreversible inhibition caused by a nucleophilic substitution of one aromatic nitro group by the thiolate of Cys122. Other analogues with poorer initial binding did not possess the irreversible component of binding.

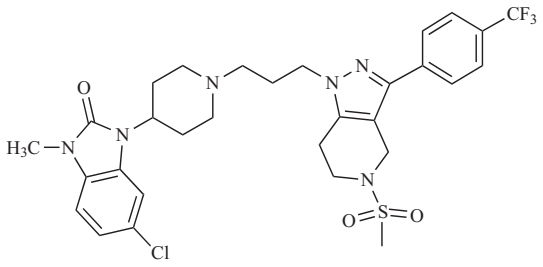
Fattorusso *et al.* [150] have discovered a series of Caspase inhibitors based on a combination of virtual screening and NMR techniques. Screening for inhibitors of Caspase-8 by ^{19}F NMR provided the initial hit **44** (K_i 30±5 μM). 500 analogues of **44** (ChemNavigator, Asinex, Maybridge) were docked and ranked against the crystal structure of Caspase-8 using Gold and FlexX. 10 analogues were selected for testing against Caspase-8 in a fluorogenic assay with **45** being the most potent (K_i 2.7 μM). This series of compounds also exhibited similar activity against Caspase-3 and –7.



40 (IC_{50} 1 μM)

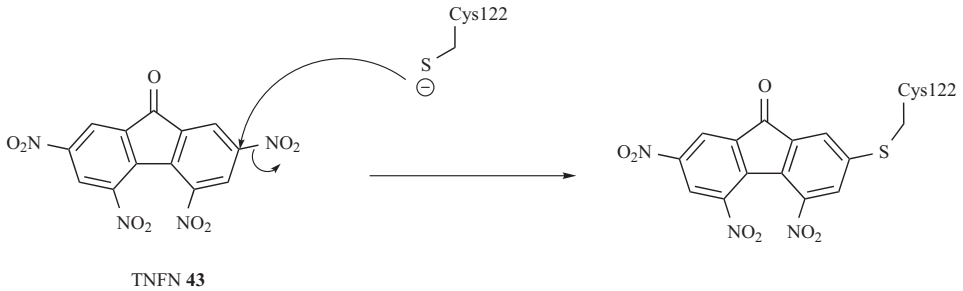


41 (IC_{50} 20 nM)



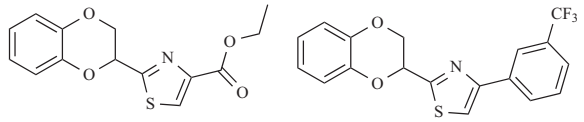
JNJ 10329670 **42** (IC_{50}100nM)

Fig. (19).



TNFN **43**

Fig. (20).

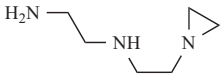


44 **45**

Fig. (21).

Metalloproteases

Virtual screening of metalloproteases has not been as widely undertaken as other classes of protease chiefly as a result of uncertainty of the mathematical models for predicting metal affinity of the ligands, and binding geometry of the metal [151] which dominates the molecular recognition in these enzyme-ligand complexes. In addition MMP's in particular [152-154] are known to undergo significant conformational changes in the S1' pocket on ligand binding. In general, protein flexibility is still poorly simulated in many of the available docking packages. Accordingly, most publications on docking and virtual screening on metalloproteases currently concentrate on ensuring [39, 41] or improving the accuracy of known ligand binding [151, 155, 156] and incorporating the protein flexibility into the equation [157], or the use of more pharmacophore-based approaches, such as for ADAM12 [158], Anthrax Lethal Factor [159], and botulinum neurotoxin A metalloprotease [156]. One report [160] of virtual screening-based discovery of novel angiotensin-converting enzyme 2 (ACE2) inhibitors from the NCI library of 140 000 compounds using DOCK 5.1 describes nine compounds chosen from the docking screen for *in vitro* assay. The most potent (IC_{50} 57±7 μM) compound **46**, an aziridine interestingly was also found to be effective in blocking the SARS coronavirus spike protein-mediated cell fusion.



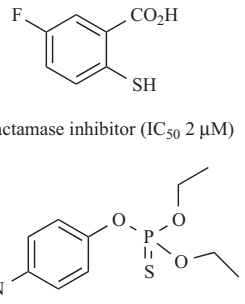
46 IC_{50} 57±7 μM

Fig. (22).

A very recent article from Irwin *et al.* [161] on metalloenzymes in general found that a modified DOCK screening protocol was capable of dealing with zinc, nickel and molybdenum, successfully discriminating known metalloprotease (MMP-3) inhibitors from the MDDR over random, although iron centres (heme) were found to be problematic. These authors then went on to successfully discover new fragment-based inhibitors of *Bacteroides fragilis* β-lactamase from the ZINC [85] database. Interestingly, they also successfully discovered new substrates for the zinc-dependent phosphotriesterase from *Pseudomonas diminuta*.

Serine Proteases

One of the earliest virtual screening studies on serine proteases was a Ludi-based hydrid fragment-based docking and structure-based de novo design project of Bohm *et al.* [162] who discovered novel thrombin inhibitors using fragments found the in the ACD. These workers also obtained an X-ray crystal structure of the lead compound **50** bound to thrombin where the amidinophenyl and distal phenoxy groups are bound as predicted from the docking into the S1 and S3 pockets.

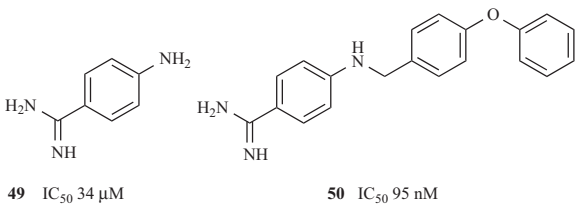


47 β-lactamase inhibitor (IC_{50} 2 μM)

parathion ethyl **48**

(phosphotriesterase substrate, k_{cat}/K_m $1.3x10^{-6}$ M

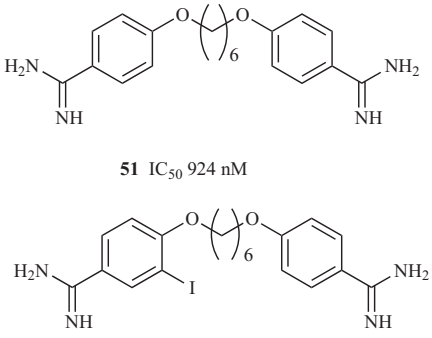
Fig. (23).



49 IC_{50} 34 μM **50** IC_{50} 95 nM

Fig. (24).

Enyedy *et al.* [163] have described a virtual screening approach to novel S1-targeted bis-benzamidine inhibitors of matriptase based on a homology model derived from human thrombin. They used DOCK and the NCI database to derive an assay set of 69 compounds from the top 200 ranked docked structures. Of the 69 compounds selected for screening 15 compounds exhibited >95% inhibition at 75 μM (Fig. **25**).



51 IC_{50} 924 nM

52 IC_{50} 191 nM

Fig. (25).

Liebeschuetz *et al.* [77] have described the use of Pro_Select to derive inhibitors **53**-**56** of Factor Xa. The workflow describes differ from many other docking approaches in that the starting libraries of virtual chemicals are designed to be synthesisable from a common template using fragments derived from databases such as the ACD, rather than being a list of commercially available compounds. They are also able to be "grown" into the target site and so the technique can also be classified as a *de novo* technique.

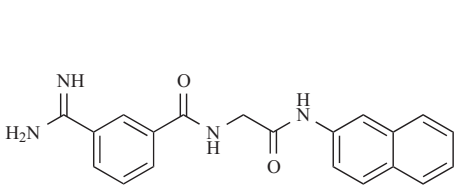
Whilst not strictly within the terms of reference of this review, a note is due to a paper from Lang *et al.* [164] who have developed a method for prefiltering compound libraries into those which possess structural features or molecular descriptors that predispose them to be hits against serine proteases.

Aspartyl Proteases

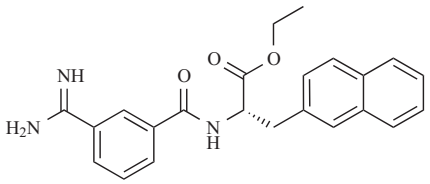
Huang *et al.* [165] have reported a fragment-based virtual screening approach to the discovery of non-peptide inhibitors of β-secretase (BACE-1). An initial virtual screen indicated that compounds such as **57** bearing a phenyl urea group could bind centrally to one or more of the catalytic aspartates. Subsequently, from a database of 6 million molecules 32 000 compounds containing a phenyl urea moiety were selected and docked into the BACE crystal structure. Of the top ranked 131 molecules 10 were purchased after visual inspection. Twelve compounds in the study had low micromolar affinity for BACE-1 (e.g. **58**) and also low micromolar EC_{50}'s in a cell-based assay.

NUCLEAR HORMONE RECEPTORS

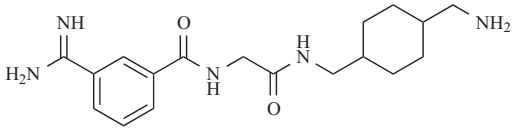
Nuclear hormone receptors are ligand-activated transcription factors [166] which bind hydrophobic endogenous ligands such as steroids, retinoids, thyroid hormone, vitamin D and prostanoids. They play a central role in normal development [167] and maintenance [168, 169] of body tissues, reproduction, and in behaviour, however they also regulate aberrant processes in diseases such as cancer [170], cardiovascular disease [171], metabolic disorders [172] such as diabetes [173] and obesity [174], and various neurological diseases [175]. The activation mechanism is a two-step process; firstly the hormone binds to the ligand-binding domain (LBD) and subsequently, this complex undergoes a change in conformation and binds to DNA upstream of the



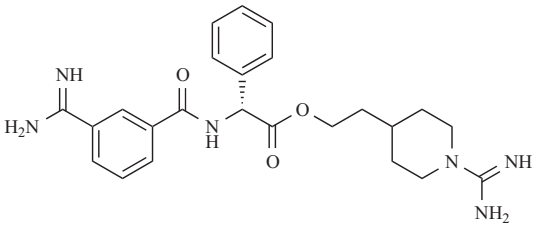
53 hit



54 lead 2 μM

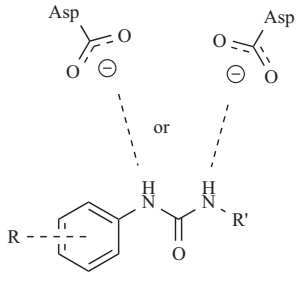


55 lead 14 μM

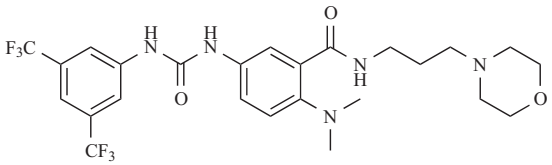


56 lead 16 nM

Fig. (26).



57



58 IC_{50} 58 μM

Fig. (27).

target gene *via* a conserved DNA binding domain (DBD) and regulation of transcription ensues. The sequence of DNA recognised by the DBD is known as the hormone response element (HRE).

Both full, partial or inverse agonist and antagonists of Nuclear Hormone Receptors are considered therapeutically interesting as drugs [176] and there are a large number of X-ray structures available for the various members of the family (Androgen, Estrogen, Pregnane X, Progesterone, PPAR, retinoid X, retinoic acid, thyroid hormone, vitamin D), many in both agonist-bound and antagonist-bound states. In 2001, 12 of the world's top 100 selling drugs targetted nuclear receptors [177].

Researchers at Molsoft (ICM software) have been prominent in the use of virtual screening against nuclear hormone receptors [31], however their research tends to examine the ability to pick known NHR ligands from a random library. However they did use ICM successfully to find two novel antagonists **59**, **60** (55% and 33% inhibition at 20 μM) of the retinoic acid receptor α from the Available Chemicals Directory (ACD) [30]. In a subsequent paper [178], these authors describe a second round of virtual screening resulting in an agonist **61** with nanomolar potency.

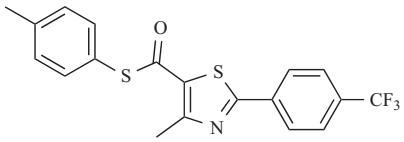
These docking results were based on a modelled antagonist-binding form of the receptor based on the raloxifene and tamoxifen-bound crystal structures of the estrogen receptor α. Later, the same group demonstrated the use of virtual screening by an identical strategy to derive 14 extremely diverse antagonists of the Thyroid hormone receptor [179] from a prefiltered version of the ACD. In this subsequent report, the initial lead compound **62** (IC_{50} 1.5 μM) was modified after virtual screening of close analogues, by chemical synthesis to generate compound **63** with submicromolar potency (IC_{50} 0.75 μM).

Virág and coworkers [180] have described a method where the FlexX-Pharm modules within the Sybyl environment was used to simultaneously dock molecules from the World Drug Index and Integrity databases into estrogen receptor α structures containing flexible regions allowing for a range of conformations spanning both agonist and antagonist binding modes. This study however did not aim to identify novel inhibitors but to examine whether known ER agonists and antagonists could be *in silico* functionally identified from the decoys. Flavone and isoflavone phyoestrogens **64**-**67** [181] have been found by virtual screening to also activate Estrogen-Related Receptor (ERRα β and γ).

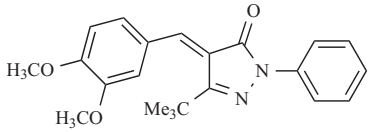
Researchers at Wyeth [182] have discovered a series of compounds (e.g. compound **68**) from the ACD *via* virtual screening using DOCK on estrogen receptor α which do not appear to bind directly to the estrogen site of the protein. Rather a secondary site, involved in the binding of SRC coactivators has been targeted. Other researchers have approached these (and other) alternative sites on NHR's for drug design, although not yet *via* virtual screening techniques [90, 183-186].

OTHER CLASSES OF TARGET

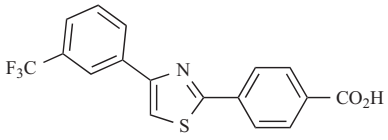
Structure-based virtual screening has also been successfully applied (Table **2**) to Kinesin [187], *Tritrichomonas foetus* Hypoxanthine-Guanine-Xanthine Phosphoribosyltransferase



59

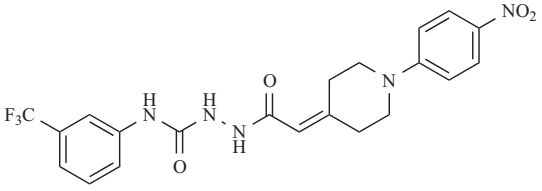


60

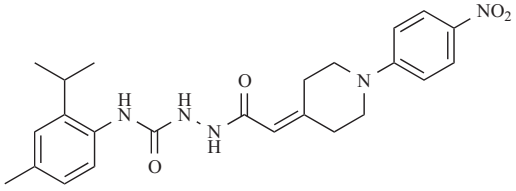


61

Fig. (28).

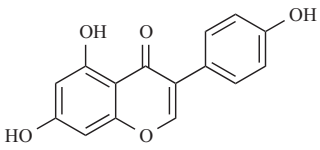


62

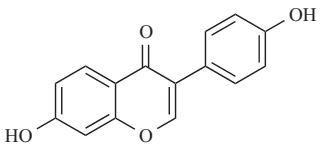


63

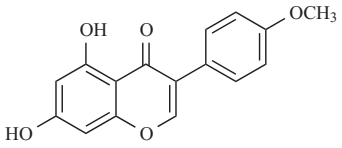
Fig. (29).



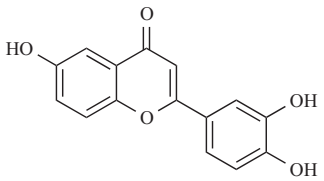
Genistein **64**



Daidzein **65**

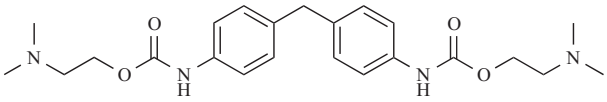


Biochanin A **66**



6,3',4' trihydroxyflavone **67**

Fig. (30).



68

Fig. (31).

(HGXPRT) [188, 189], tRNA-Guanine Transglycosylase [190, 191], Carbonic Anhydrase II [192, 193], Sex Hormone Binding Globulin [194], 5-aminoimidazole-4-carboxamide Ribonucleotide (AICAR) Transformylase [195], Peptide Deformylase [196], Protozoal Bifunctional Thymidylate Synthase-Dihydrofolate Reductase [197] , *Lactobacillus casei* Thymidylate Synthase [198], Cyclooxygenase-1 and 2 [199], Inosine 5-monophosphate dehydrogenase (IMPDH) [200], FK506 Binding Protein (FKBP) [201], Protein Tyrosine Phosphatase 1B (PTP1B) [202, 203], DNA-Ligase from *Mycobacterium tuberculosis* [204], Potassium Channel Blockers [205], Aldose Reductase [206-208], Farnesyl Transferase [209], AmpC beta-lactamase [210], Dihydrodipicolinate reductase from *Mycobacterium tuberculosis* [14], *Plasmodium falciparum* dihydrofolate reductase inhibitors [211], and *Giardia lamblia* Guanine Phosphoribosyltransferase [212]. In comparison, comparatively little research has been done on virtual screening against DNA [213] and RNA [18, 214, 215] targets in recent years, although some early success was reported [216, 217].

EMERGING TRENDS AND CURRENT LIMITATIONS

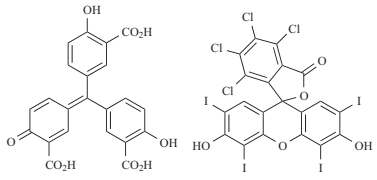
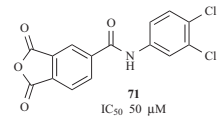
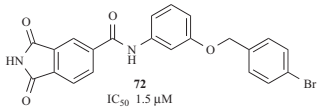
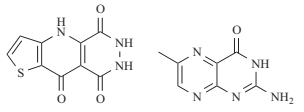
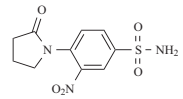
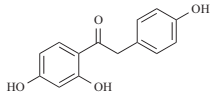
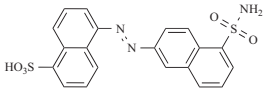
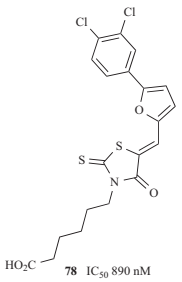
Compound Prefiltering

An emerging trend is the move away from "brute force" docking of hundreds of thousands to millions of compounds to a more rational approach based on prefiltering. Many, if not most of the successful lead-finding programs covered in this review have utilised prefiltering to some extent. Many groups have reported their own approaches to creating and utilising druglike [218-220] subsets of compounds. Publicly accessible ligand databases such as Zinc are already available as druglike, leadlike, "greasy leads" and "big'n'greasy" subsets [85].

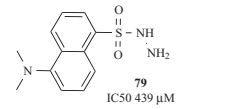
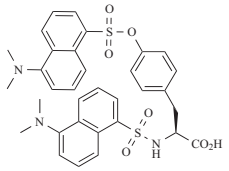
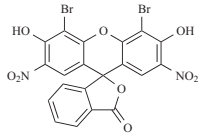
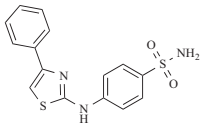
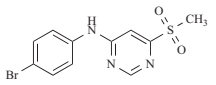
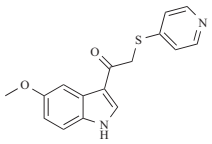
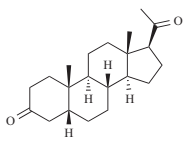
Prefiltering techniques range from simply removing classes of undesirable compounds (e.g metal complexes) to more subtle 2D [221, 222] and 3D [10, 86] similarity-based and pharmacophore searching techniques. Several successful examples discussed above use some form of hybrid or combined approaches [96, 97, 123, 165, 190-194, 208, 211].

The reduced subsets of compounds (100's to 1000's) which possess some or all of the correct features for binding, can then be docked in a more rigorous manner. This approach is more time-consuming in the second step but is being done on a subset more likely to contain true binders. Unfortunately, this method will likely produce many false negatives: those compounds which do not bind in the generally accepted way, but rather by some novel mechanism. An additional complication from using similarity-based prefiltering is that the resulting compounds may not be sufficiently novel from a patentability standpoint. Conversely a rapid low-bar docking-based prefilter to eliminate which compounds do not fit into an active site can be followed by a more detailed 3D- pharmacophore search. This technique suffers from the drawback that compounds that "just" fail

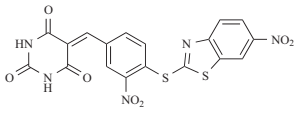
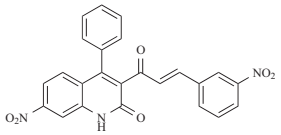
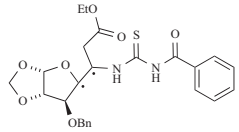
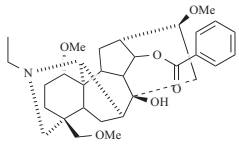
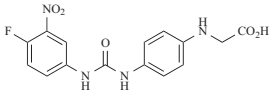
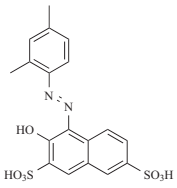
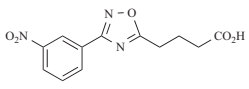
Table 2. Other Drug Targets Approached *via* Virtual Screening and Hits/Leads Obtained

Target	a) Method b) Target Structure c) Database(s) d) Programs used	Lead	Ref.
Kinesin	a) Docking b) Crystal structure c) ACD d) DOCK	 **69** IC_{50} 1.4 μM **70** IC_{50} 7.5 μM	[187]
HGXPRT	a) Docking b) Crystal structure c) ACD, then a virtual library d) DOCK	 **71** IC_{50} 50 μM  **72** IC_{50} 1.5 μM	Hit [188] lead [189]
tRNA-Guanine Transglycosylase	a) Hybrid b) Homology model c) Novo Nordisk corporate collection and commercial databases d) Unity / FlexX	 **73** Ki 5 μM **74** Ki 250 nM	[190], [191]
Carbonic Anhydrase II	a) Hybrid b) Crystal structure c) Maybridge and Leadquest DB's d) Unity / FlexS/ FlexX	 **75** IC_{50} 0.6 nM	[192], [193]
Sex Hormone Binding Globulin	a) Hybrid b) Crystal structure c) Natural product databases d) GLIDE / CATALYST	 **76** IC_{50} 14 μM	[194]
AICAR Transformylase	a) Docking b) Crystal structure c) NCI Diversity set d) Autodock	 **77** Ki 154 nM	[195]
Soybean Peptide Deformylase	a) High Throughput Screening and Docking b) Homology model c) DuPont crop protection compound library and vendor databases d) ICM	 **78** IC_{50} 890 nM	[196]

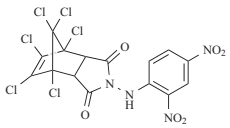
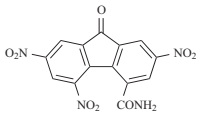
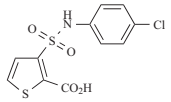
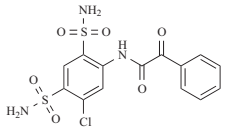
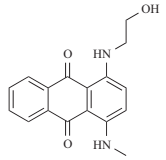
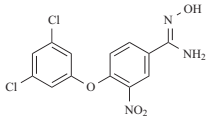
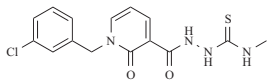
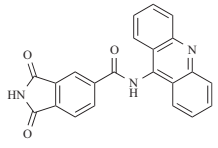
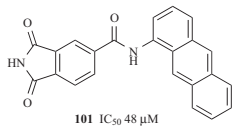
(Table 2. Contd….)

Target	a) Method b) Target Structure c) Database(s) d) Programs used	Lead	Ref.
L.Casei Thymidylate Synthase	a) Docking and analogue synthesis b) Crystal structure c) ACD d) DOCK	 **79** IC50 439 µM  **80** Ki 1.3 µM	[198]
Protozoal Bifunctional TS-DHFR	a) Docking b) Crystal structure c) ACD Database d) Dock / NWU-DOCK	 Eosin B **81** IC_{50} 100 µM	[197]
COX-1,2	a) Docking b) Crystal structure c) NCI, ChemBridge and NCI databases d) DOCK	 **82** 61% inhibition of Cox-1 at 1 µM 29% inhibition of Cox-2 at 1 µM  **83** 14% inhibition of Cox-1 at 1 µM 60% inhibition of Cox-2 at 1 µM	[199]
IMPDH	a) Fragment Docking b) 2x in-house Crystal structures c) Small molecule Roche in-house reagent library d) FlexX	 **84** IC_{50} 32 µM	[200]
FKBP	a) Docking b) Crystal structure c) ACD and CCD databases d) SANDOCK	 **85** Kd 7 µM 5b-Pregnan-3,20-dione	[201]

(Table 2. Contd….)

Target	**a) Method** **b) Target Structure** **c) Database(s)** **d) Programs used**	**Lead**	**Ref.**
PTP1B	a) High Throughput Screening and Docking b) Crystal Structure c) ACD, Biospecs, Maybridge d) DOCK / NWU-DOCK	 **87** IC_{50} 4.1 μM  **86** K_i 54 μM	[202, 203]
DNA Ligase	a) Docking b) Crystal structure c) In-house database d) Autodock / Gold	 **88** IC_{50} 4 μM stereochemistry undefined	[204]
K+ Channel Blockers	a) Docking b) Homology Model c) China Natural Products Database d) DOCK / Sybyl / Autodock	 **89** IC_{50} 4 μM	[205]
Aldose Reductase	a) Docking[206],[207] / Hybrid[208] b) Crystal Structure c) ACD[206, 207] / Maybridge / Bionet[208] d) ADAMA&EVE[206] DOCK[207] Unity / FlexX[208]	 **90** IC_{50} 6 μM  **91** IC_{50} 0.6 μM  **92** IC_{50} 2.4 μM	[206] [207] [208]

(Table 2. Contd….)

Target	a) Method b) Target Structure c) Database(s) d) Programs used	Lead	Ref.
Farnesyl Transferase	a) Docking b) Crystal Structure c) ACD d) EUDOCK	 **93** IC_{50} 25 μM  **94** IC_{50} 80 μM	[209]
AmpC beta-lactamase	a) Docking b) Crystal Structures c) ACD d) NWU-DOCK	 **95** K_i 26 μM	[210]
DHDP reductase	a) Combined HTS and Docking b) Crystal structure of e.coli DHDP reductase c) Merck chemical collection d) FLOG	 L-586,078 **96** Ki 42 μM	[14]
TAR RNA	a) Docking b) Homology model c) ACD d) DOCK / ICM	 **97** CD_{50} 1 μM	[214]
Plasmodium falciparum DHFR	a) Hybrid docking/pharmacophore b) Crystal structure c) ACD d) Catalyst / Dock3.5	 **98** K_i 2.4 μM  **99** K_i 11.3 μM	[211]
Giardia lamblia GPRT	a) Docking b) Crystal structure c) In-house database d) Dock	 **100** IC_{50} 40 μM  **101** IC_{50} 48 μM	[212]

steric matching criteria may in fact turn out to be tight true binders to due relatively minor changes in receptor side-chain conformations.

Consensus Scoring

Consensus scoring is an approach where different scoring functions from different programs are applied to a set of docking results in an attempt to find "the common ground", i.e. which compounds consistently rank highly across different algorithmic approaches. Consensus scoring has been in use for over 5 years, [223, 224] and is now generally considered a mainstream approach. As more research into docking and scoring validation is published it is becoming clear that the use of single empirical scoring functions based on a detailed analysis of a well-studied subset of protein-ligand crystal structures cannot correctly identify the most potent of a collection of similar ligands if that class of *proteins and ligands* has never previously been part of the test set [71]. To counteract this obvious failing, it is necessary to use as diverse a range of scoring functions as possible, which have been derived from different test sets, in order to average out errors and biases. Given sufficient diversity, 3 to 4 scoring functions has been reported to be sufficient for effective consensus scoring [225]. Kontoyianni *et al*. have recently demonstrated that the nature of the active site under investigation (hydrophilic-partly hydrophobic-hydrophobic) can in some cases be correlated with the success or failure of a particular scoring function to rank compounds consistently [59].

Flexible Receptor Binding

There can be no doubt that a major limitation of the current methods of docking ligands has been the use of rigid receptor models. In the same way that Fisher's original lock and key paradigm [226] gave way to Koshland's induced fit hypothesis, [227] it is clear that (incorrectly) treating the receptor as a rigid entity must give way to methods explicitly dealing with receptor flexibility, and thus addressing the induced fit problem. To be fair however this has more to do with the historic computational cost of including flexibility as a variable than any scientific naivete. This shortcoming of the virtual screening method is quite target dependant. For example, protein flexibility is more of an issue for enzymes such as aldose reductase which can accommodate a wide range of substrates, compared with serine proteases such as trypsin and Factor Xa [132] which are heavily pre-organised for ligand binding. An additional difficulty is the change in structure of cofactor-associated enzymes such as Factor VIIA, which exhibits significant changes in active site structure when the essential cofactor (tissue factor) is bound. [228, 229] This will most likely extend also to several other classes of drug targets, where the active species are assemblies of proteins. Newer methods of incorporating protein flexibility into the virtual screening protocols are appearing, [131, 230-232] however they are also not without their own limitations [233]. If flexibility cannot be taken into account, it is necessary to choose the target structure carefully. In one study [234] researchers prepared homology models of 10 proteins for which there were both apo and ligand-bound structures available in the PDB and compared docking results on all 3 forms of the protein. In 8 cases they considered the bound form to be suitable for generating reliable docking results, in only 3 was the modeled conformation considered suitable.

It is also becoming increasingly apparent that for reasons of computational cost, most docking packages using in high-throughput mode do not sufficiently or rigorously sample the conformational space of the ligands as well [54, 71, 78, 235]. In addition, many of the docking packages do not give adequate time to full minimisation of the ligands in the active sites resulting in artificially high internal energy penalty scores hence low rankings. It has also been noted that frequently, bound conformations of flexible ligands do not neccessarily correspond to low energy solution conformations [236, 237]. In order to overcome this, it is becoming increasingly common to take a number of relatively well ranking hits, and allow a more (computationally expensive) rigorous minimisation to better evaluate their binding modes and obtain more reliable scoring; e.g. Huang *et al.* [116]. An alternative approach to avoiding the issue of flexibility is to undertake fragment-based docking, rather than docking larger flexible molecules, often targeting one pocket at a time [165, 238, 239].

Other current limitations of the method which are still under constant development are the inadequate treatment of water, both as the biological solvent in question, and also an explicit partner in ligand binding, [132, 240, 241] the nearly absolute requirement for visual inspection at some stage in the process, [68, 123, 132] inadequate realisation of the frequency with which the protonation states of ligands and receptors can change on binding due to localised pKa effects, [132] and an inadequate treatment of tautomerisation [242]. For example, water molecules constitute an important part of the ligand-protein binding process in approximately two-thirds of the entries in Relibase, [132] and famously the Watson-Crick model of DNA was flawed until the problem of the keto-enol tautomerisation of thymine and guanine was recognized [243, 244]. Within these limitations, general consensus of the field at present suggests that the docking component of the virtual screening protocol is working quite well, but that the scoring techniques available to us today still have a way to go before they can be truly predictive [39, 66].

CONCLUSIONS

Although virtual screening approaches to drug discovery have become increasingly popular over the last 5 years there are a number of significant limitations and impediments to its implementation in successful discovery of new drug leads. It cannot therefore yet be termed a mature science, [132] one capable of accurately predicting the affinities of diverse structures, or indeed to discriminate in favour of the best of a closely related series of compounds.

In spite of the limitations discussed above, virtual screening is still being enthusiastically embraced by the pharmaceutical industry and academia alike, if not quite to the extent suggested by the early hype surrounding HTS and combinatorial chemistry. For the pharmaceutical industry, which has already invested heavily in the technology and in

personnel needed for effective computer-aided drug design, virtual screening does have some important advantages in speed and cost in comparison to established methodologies such as high throughput screening. In an academic setting one clear advantage of the technology is that research groups, whose principal focus is not on chemistry but rather biology, can use the technology easily to find lead molecules for their protein of interest. Armed with a lead molecule, and often a novel target, these academic groups will be better able to engage chemists to tackle the pharmaceutical challenges in the decades ahead.

ABBREVIATIONS

5HT1a = 5-Hydroxytryptamine,Serotonin

ACD = Available Chemicals Directory

ACE = Angiotensin Converting Enzyme

ADAM12 = A disintegrin and metalloprotease

ADP = Adenosine diphosphate

AICAR = 5-aminoimidazole-4-carboxamide Ribonucleotide

Akt = Protein kinase B

ATP = Adenosine triphosphate

BACE = β-secretase

CCD = Cambridge Crystallographic Database

CCR3 = Chemokine Recptor 3

CDK = Cyclin Dependant Kinase

Chk-1 = Checkpoint Kinase-1

CMC. = Comprehensive Medicinal Chemistry

CK2 = Casein kinase II

CML = Chronic myelogenous leukemia

COX = Cyclooxygenase

D2R = Dopamine Receptor 2

DBD = DNA binding domain

DHFR = Dihydrofolate Reductase

DHPR = Dihydrodipicolinate reductase

ELISA = Enzyme Linked Immuno Sorbant Assay

EphB2 = Erythropoietin-producing hepatocellular B2 Tyrosine kinase

Tyrosine kinase

ER = Estrogen Receptor

ERRα β and γ = Estrogen-Related Receptor α β and γ

FAC-MS = Frontal Affinity Chromatography interfaced to Mass Spectrometry

FKBP = FK506 Binding Protein

GPCR = G Protein-Coupled Receptor

GPRT = Guanine Phosphoribosyltransferasem

hAVCP = Human adenovirus cysteine proteinase

HGXPRT = Hypoxanthine-Guanine-Xanthine Phosphoribosyltransferase

HIV = Human Immunodeficiency Virus

HRE = Hormone response element

HTS = High-throughput screening

IMPDH = Inosine 5-monophosphate dehydrogenase

LBD = Ligand-binding domain

MDDR = Medical Drug Data report

MMP = Matrix Metalloprotease

MOBILE = Modeling Binding Sites Including Ligand Information Explicitly

NCI = National Cancer Institute

NHR = Nuclear Hormone Receptor

NK1 = Neurokinin Receptor 1

NMR = Nuclear Magnetic Resonance

P2X = Purine/ pyrimidine-gated ion channels

P2Y = Purine/ pyrimidine activated G-protein coupled receptors

p56 Lck = Lymphoid T Cell Tyrosine Kinase

PDB = Protein Databank

PPAR = Peroxisome-Proliferator-Activated Receptor

PRPP = 5-Phosphoribosyl-1-pyrophosphate

PTP1B = Protein Tyrosine Phosphatase 1B

SARS = Severe Acute Respiratory Syndrome

VEGFR = Vascular Endothelial Growth Factor Receptor

WDI = World Drug Index

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