In Search for New Leads

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Pharmaceutical industry is short of new drugs. Whereas in past decades about 50-60 new drugs (new chemical entities, NCEs) were approved every year and introduced into therapy, this number declined significantly in the last few years, reaching its historical low in the year 2000 with 27 NCE's, 2001 with 24 NCEs, and 2002 with only 18 NCEs approved by the FDA [1]. Correspondingly, research costs for a new drug are estimated to be in the US-$ 500-900 millions (cf. e.g. [2]). However, considering all failures in drug research and comparing worldwide research and development costs for new drugs (including biologicals) of about US-$ 45 billions (estimated for 2001) with the number of NCEs, this figure might be even higher.

The decline in the number of new drugs has quite different reasons (cf., e.g. [3-5]). The two most important ones seem to be an already achieved high therapeutic standard in many indications, focusing research now on chronic degenerative and other fatal diseases, like coronary heart disease, Alzheimer's disease, arthritis, cancer, and AIDS, as well as enhanced regulatory requirements for efficacy and safety of new drugs. However, the current situation reflects also a shortage of new lead structures that can be optimized into therapeutically useful drugs. Correspondingly, this overview describes and evaluates different strategies in the search for new leads.

What is a lead?

Many attempts have been made to define the properties that characterize a lead structure. First of all, the compound must have some desirable biological activity, although it may be weak and even non-selective. There must be related analogs, indicating that structural modification will modulate biological activity as well as other properties. The lead structure must not be an extremely polar or lipophilic compound which may cause problems in bioavailability; it should not contain toxic groups or groups that will produce toxic metabolites. It should not irreversibly react with its biological target (although one has to admit that some most successful drugs, like acetylsalicylic acid, the penicillins, and omeprazole are indeed irreversible enzyme inhibitors).

Most important for the successful optimization of a lead structure to an active, selective, orally bioavailable, and non-toxic drug seem to be a certain molecular weight and lipophilicity range. Lead structure optimization is an evolutionary procedure, in which every minor or major improvement in certain properties leads to a new analog, which is further optimized until the final candidate has all desired properties to start its clinical investigation. Experience shows that drug candidates most often become larger in size and more lipophilic in this process [6-12]. Thus, a recommendation has been that a lead should have a molecular weight < 350 and a lipophilicity, expressed by log P (P = n-octanol/water partition coefficient), smaller than 3 [6]. On the other hand, the Lipinski "rule of five" demands that drugs should have a molecular weight < 500, a lipophilicity range of log P < 5, no more than 5 hydrogen bond donors, and no more than 10 N and O atoms (a rough estimate of the number of hydrogen bond acceptors) in the molecule [13]; there is a high risk of poor bioavailability if two or more of these conditions are violated. Other groups explored polar surface area as a factor determining bioavailability [14,15], as well as the flexibility of a molecule, expressed by the number of rotatable bonds [16]. The molecular properties of marketed drugs and clinical candidates have been investigated by several groups [17-19].

Hann et al. [8,9] compared 470 lead/drug pairs from Sneaders book on the exploitation of drug prototypes [20]. They showed that the average molecular weight increase of a lead to the final drug was only 38 mass units (63 mass units

for the 78% drugs that had a higher molecular weight than their original lead) [8]. However, the chemical variation of complex natural products, like morphine, quinine, or the curare alkaloids, demonstrates that much simpler analogs can be derived which retain the biological activity of the original lead. The following discussion will provide evidence that there are many exceptions to the empirical definitions of the lead structure properties, which are listed above, and that in special cases even "bad" leads can be successfully optimized to valuable drugs.

Natural products as traditional sources of lead structures

Natural products have been the richest source of drugs and lead structures (e.g. [21-24]). About half of our drugs are still natural products, derivatives, or analogs of natural products. Whereas, in the past, plant products played a predominant role and microorganisms were only investigated as producers of antibiotics, nowadays several important classes of drugs are extracted or derived from microorganisms.

It all started with foxglove, morphine, quinine, and salicylic acid. Cardiac glycosides, including analogs with improved pharmacokinetic properties, were extracted and derived from Digitalis species and other plants. Morphine turned out to be a valuable lead for major analgesics, some of them with much simpler chemical structures, antitussives, morphine antagonists, obstipants, and neuroleptics. Also in the case of quinine, much simpler analogs could be derived from this complex natural product. Salicylic acid is a natural product with weak antiinflammatory activity; its derivative acetylsalicylic acid acts as an irreversible inhibitor of cyclooxygenase, making the compound more active and also suited for the prophylaxis of thrombotic diseases. Further plant products that served as leads in drug research are e.g. the curare alkaloids, papaverine, atropine, and cocaine (see textbooks of drug discovery and medicinal chemistry, e.g. [20,25,26]). The antitumor drugs taxol and camptothecin, the anti-Alzheimer natural product huperzine, and the antimalarial drug artemisine are recent examples of plant products of therapeutical interest.

With the exception of epibatidine and some peptides, like teprotide, hirudin, and the conotoxins, animal toxins are more important as pharmacological tools (e.g. tetrodotoxin) than as therapeutics or lead structures (for the role of endogenous neurotransmitters, steroids, etc., see below).

Since 1928, when Sir Alexander Fleming discovered the lysis of bacteria by a secretion product of a Penicillium strain, microorganisms have been a rich source of antibiotics. The original penicillin structure has been optimized, step by step, to bioavailable analogs, to broad spectrum antibiotics, and finally to lactamase-resistant derivatives. In addition to penicillin, the cephalosporins, tetracyclins, chloramphenicol, streptomycin, rifampicin, valinomycin, etc., turned out to be valuable lead structures or antibiotic drugs themselves. But not only antibiotics resulted from microorganisms, also cardiovascular drugs and the hallucinogenic lysergic acid diethylamide (Lysergide, LSD) from ergot (Secale cornutum), the immunosuppressants cyclosporin A and tacrolimus, the antitumor principle epothilone, and the most important group of cholesterol biosynthesis-blocking statins. Also the anticoagulant coumarins, like phenprocoumon and warfarin, were derived from dicoumarol, a microbial product first isolated from rotten hay [23].

Serendipitous drug discoveries

Some of the very first drugs were discovered by serendipity, already 150 years ago [20,27-31]. The use of nitrous oxide and ether as narcotic gases in surgery resulted from the observation that persons which inhaled these chemicals in fun parties did not experience any pain after being injured. The vasodilatory activity of amyl nitrite and nitroglycerin was also discovered by accident; chemists working with these organic nitrites experienced strong headache after inhaling or ingesting minor amounts. Some other drugs resulted from wrong working hypotheses, e.g. chloral hydrate, which was supposed to degrade metabolically to the narcotic chloroform (indeed the metabolite trichloroethanol is the active form), and urethane, which was supposed to release ethanol but is a hypnotic itself. Acetylsalicylic acid was considered to be just a better tolerable derivative, a prodrug, of salicylic acid but it turned out to have a unique mechanism of action (see above). Phenolphthalein was considered to label cheap wines; in a heroic self-experiment, a pharmacologist experienced its drastic diarrhoic activity. A secretary fell asleep for about 20 hours after the first human application of clonidine, which was supposed to be a nasal congestant but turned out to be a strong antihypertensive drug. The sto-

ries of the serendipitous discoveries of penicillin, LSD and the first tranquilizer, chlordiazepoxide, are well known [20,31]. The anticoagulants of the dicoumarol type resulted from the observation that cattle bled to death after being fed with rotten hay. The anticoagulant warfarin was originally used as a rat poison; its clinical applicability was confirmed, when a US soldier tried to commit suicide but survived. Nowadays this "rat poison" is a most valuable drug in the prevention therapy after stroke and other thrombotic diseases. All major artificial sweeteners, i.e. saccharin, cyclamate and aspartame, were serendipitous discoveries. Chemists experienced the sweet taste when licking their fingers or smoking a cigarette [30].

A closer inspection of drug discovery stories shows that serendipity and sagacity played an important role in many cases [20,27,31]. Fleming might have discarded his spoiled bacteria culture and Sternbach might have neglected the crystals of chlordiazepoxide when he cleaned up his laboratory. But they didn't because they were experienced investigators, according to the formulations "chance only favors the prepared mind" by Louis Pasteur and "discovery consists of seeing what everybody else has seen and thinking what nobody else has thought" by Albert Szent-Györgi, the discoverer of vitamin C.

Rational approaches - the golden age of drug research

Besides natural products from plants, endogenous neurotransmitters and steroid hormones have been the richest source of new drugs. From the elucidation of the biochemical mechanisms underlying the transmission of nerve impulses and the deeper understanding of hormone effects, a large number of therapeutically useful drugs resulted, not only receptor agonists but also antagonists. This phase of drug research may be considered as its golden age [32]. Nearly every modification of dopamine, serotonin, histamine, or acetylcholine, using the modification strategies of classical medicinal chemistry [33], resulted in a compound with modified activity and selectivity, most often in a drug candidate. A broad repertoire of drugs, some of them still being used today, resulted from this period of the 1950's and 1960's [20,26,27,34]. The very first H1-antihistaminic drug, diphenhydramine, today considered to be obsolete due to its sedative side effect, was synthesized in the mid-40's of the last century by a young university professor. Immediately, antihistaminic drugs became popular as miracle drugs. By serendipity, it was also discovered that dimenhydrinate, the complex of 8-chlorotheophylline with diphenhydramine, is an efficient drug against travel sickness; its "clinical trial" happened in 1947, in a sailing of the ship "General Ballou" from New York to Bremerhaven [20,28]. Diphenhydramine became such a financial success that the royalties for the inventor of this compound exceeded the income of the president of the company Parke Davis, which distributed the drug; later this inventor became its Director of Research [20,28]. Still today, the potential of neurotransmitter agonists and antagonists, e.g. of 5-HT receptor ligands, and of neurotransmitter uptake inhibitors has not been fully exploited.

Similar success stories can be told about the steroid hormones and their more selective synthetic analogs. A first breakthrough in the development of bioavailable analogs resulted from the introduction of 17a-residues, especially the ethinyl group, into estrogenic, gestagenic and androgenic steroids, in order to avoid the rapid metabolic conversion of 17-keto or 17b-hydroxy groups into inactive 17a-hydroxy compounds. Synthetic corticosteroid analogs were enthusiastically appreciated as another group of miracle drugs, when arthritic patients immediately got relief from their chronic pain. Only later it was realized that this benefit is to some extent counterbalanced by serious side effects, especially in their chronic application. Less well known is the history of the first ovulation blocker, norethynodrel, developed by Searle in the late fifties of the last century. Whereas the design of this analog as a potent, orally bioavailable gestagen followed a rational principle, the final drug was based on a serendipitous observation. Its efficacy to avoid any undesired pregnancies resulted only from the fact that the synthesis started from mestranol, the methyl ether prodrug of the potent estrogen ethinylestradiol. First batches, used in the clinical trials, contained a minor amount of this starting material. When Searle was going to introduce the drug to the market, they decided to produce norethynodrel in pure form. However, immediately pregnancies resulted from the new batches. Searle was forced to supplement the estrogenic "impurity", making the combination of both compounds as safe as before [20]. The development of ovulation blockers might have been retarded by years or even decades without this unintentional investigation of a gestagen/estrogen combination. Unfortunately, the estrogen amount of the first

generation of ovulation blockers was too high - severe thrombotic side effects resulted in many cases.

In recent years, many enzyme inhibitors were developed from leads that mimic the transition state of the corresponding enzyme. Protease inhibitors [35] start from cleavage-site peptides, where the involved amide bond is converted into another functionality. Experience shows that serine and cysteine protease inhibitors should contain the P-1, P-2, etc., amino acids (the "amino-terminal" peptide), sometimes combined with a carboxyl group modification that is capable to interact with the catalytic serine or cysteine, e.g. an aldehyde, activated ketone, chloromethyl ketone, or boronic acid. Metalloprotease inhibitors, on the other hand, should contain the P-1', P-2', etc., amino acids at the "carboxy-terminal" side, with a metal-chelating group instead of the amino group of this peptide, e.g. a sulfhydryl group, iminoacetic acid, or hydroxamic acid. The situation is again different for aspartyl protease inhibitors: the amino acids at both sides of the cleavable peptide bond need to be conserved and this peptide bond has to be replaced by an enzymatically stable isoster, preferentially of the transition state [35]. The problems of the conversion of such peptides into non-peptidic analogs are discussed below.

"Me too" research

Copying existing drugs, with only minor chemical variations, is designated as "me too" research. Whereas the marketing of analogs without major therapeutic advantages does not promise any benefit, many examples demonstrate that later analogs show indeed major advantages, like the bioavailable, broad-spectrum, and lactamase-resistant penicillines (see above), the diuretic and antidiabetic sulfonamides that were derived from antibacterial sulfonamides (see later section), polar H1 antihistaminics without sedative side effects, or β1-specific antagonists as well as partial agonists, with and without α1-antagonistic activity, as compared to the original nonspecific β1- and β2-inhibiting betablockers. Sometimes a second drug in the market has some therapeutic advantage that immediately puts it in first place, e.g. ranitidine vs. cimetidine or enalapril vs. captopril. Despite the chances of improvement of an existent drug, "me too" research is nowadays only performed if blockbuster drugs may result, like uptake-inhibiting antidepressants [36], statins [37], or PDE5 inhibitors [38,39]. Not "me too" is the goal of pharmaceutical industry, but "me better", "me first" or even "me only".

Peptides to peptidomimetics

Many substrates of enzymes, e.g. angiotensinogen, angiotensin, fibrinogen (as a precursor of fibrin), HIV GAG and GAG-POL proteins (the precursor proteins of HIV protease and other HIV proteins), and many enzyme and receptor ligands, e.g. the serpins, enkephalins, neurokinins, somatostatin, fibrinogen (as a GP IIb/IIIa receptor ligand), vitronectin, etc., are either peptides or proteins. In contrast to protein-protein interactions in signaling chains, the interaction of these ligands with their target is often mediated by only a few amino acid side chains. The rest of the polypeptide or protein stabilizes a certain 3D conformation of this part of the molecule; the RGD (arginine, glycine, aspartate) motif, which interacts with different integrins in (obviously) different conformations, is a striking example.

Peptides can easily be synthesized in large number - even millions or billions different analogs are no problem, if parallel synthesis is used to produce mixtures of analogs. Correspondingly high-affinity substrates or ligands can be discovered in short time. However, the next step, the chemical conversion of such a peptide lead into a non-peptidic ("peptidomimetic"), bioavailable drug is far from being trivial. Several partial structures have been proposed to mimic peptide loops, the preferred 3D structural motif that interacts with other proteins. However, with the exception of the promiscuous benzodiazepines, most other scaffolds are described in literature but have not yet been converted into active analogs.

In the case of morphine and its many analogs, no conversion of the enkephalin peptides to this complex natural product has been performed because morphine was first. Despite some modeling attempts, to prove the "pharmacophoric similarity" between enkephalins and morphine, one must conclude that the synthesis of morphine would have never been achieved, just from the structure of these pentapeptides. An example, where this has been successfully performed, are some integrin ligands. First, some cyclic peptides showed selectivity for certain integrin receptors [40] and finally benzodiazepine peptidomimetics with enormous selectivities resulted [41,42]. Other cases of the successful conversion of peptides into peptidomimetics are neurokinin-1 and -2 receptor

ligands [43,44] and somatostatin receptor ligands with pronounced receptor subtype selectivity [45].

Also in the case of HIV protease inhibitors, several peptidomimetic drugs could be derived from the sequence of the cleavage site [46]. The first HIV drugs saquinavir, ritonavir, and indinavir still look very much like peptides, whereas the later analogs nelfinavir, amprenavir, derived by structure-based design, as well as the DuPont inhibitors (not yet marketed) [47,48], are real peptidomimetics. However, the world-wide capital spending to arrive at these drugs must have been in the US-$ billions. Many companies put the very same effort into the development of non-peptidic, orally available renin and thrombin inhibitors, without much success. Thus, the conversion of peptides into peptidomimetics is possible; it has indeed produced some success stories but it cannot be considered to be a straightforward, generally applicable strategy.

The optimization of drug side effects

Most drugs show, in addition to their main mechanism of action, some side effects. For therapeutic use these side effects must be tolerable, considering the expected benefit from the drug treatment. In drug discovery, such side effects have often paved the way to applications in a different indication. A very first example were mercury organomercurials (now being obsolete), which were originally used for the treatment of syphilis but turned out to act as diuretics. Alternative drugs that are still used today resulted from the optimization of the diuretic side effects of antibacterial sulfonamides. After the observation of severe hypoglycemic effects in patients, leading even to death cases, by another antibacterial sulfonamide, antidiabetic drugs were developed from these leads. The antitussive and obstipant side effects of morphine could be optimized to non-narcotic antitussives (several of them belonging to the enantiomeric series of morphine analogs) and non-narcotic antidiarrhoics. Iproniazid, the N-isopropyl analog of the tuberculostatic drug isoniazid, turned out to be an antidepressant, when clinically investigated as a potential antituberculous drug in some depressive patients. The very first neuroleptic chlorpromazine, a dopamine antagonist, was developed from the antihistaminic drug promethazine; surprisingly, the close analogs imipramine and desipramine are antidepressants because of their neurotransmitter uptake inhibition; thus, different mechanisms of action and completely different therapeutic applications may result from minor structural differences (for reviews of such drug developments, see e.g. [20,27,28]). Acetylsalicylic acid was used for nearly a century as a mild analgesic and antipyretic drug before its mechanism of action was discovered. When it turned out to irreversibly inhibit platelet cyclooxygenase (in contrast to other cells, platelets are unable to synthesize cyclooxygenase), its value for the prophylaxis of stroke and other thrombotic diseases was recognized.

Two prominent examples of the "use" of a drug side effect for therapy, from our time, should be mentioned. The first drug for the treatment of male sexual disorder, sildenafil (Viagra, Pfizer), resulted from the optimization and development of antiallergic, antihypertensive, and antianginal drug candidates; in a tolerance study in man, a surprising side effect of strengthening penile erections showed up, which finally led to the development of sildenafil in this therapeutic direction [49]. The second example is the antileukemic drug imatinib [50]. In more than 90% of all patients with chronic myelogenous leukemia (CML), a crossover between chromosomes 9 and 22 produces a shorter version 22 (the Philadelphia chromosome), which codes for a new protein, the so-called bcr-abl protein kinase, a constitutionally active tyrosine protein kinase. At Novartis, structural modification of a protein kinase C (PKC) inhibitor produced analogs that were also inhibitors of bcr-abl kinase. Then, a minor chemical modification, the introduction of a methyl group in a certain position, abolished the undesired PKC activity; further optimization led to the better soluble, bcr-abl kinase-specific analog imatinib (Gleevec,, Glivec,; Novartis), which is the very first cure for CML [50].

Some classes of compounds belong to so-called "privileged structures" [51,52], producing drugs with many different activities, e.g. benzodiazepines, which can be tranquilizers (i.e. GABA receptor agonists), GABA receptor antagonists and inverse agonists, opiate receptor agonists, CCK receptor, NK-1 receptor, vasopressin and integrin receptor antagonists, farnesyl transferase inhibitors, potassium channel modulators, muscle relaxants, hypnotics, neuroleptics, and antidepressants. Recently, Wermuth has proposed to apply the "selective optimization of side activities" (the SOSA approach) as a general strategy in drug discovery [53]. Examples include, inter alia, the conversion of a b-blocker prototype into the

potassium channel opener cromakalim [54], and the optimization of some side activities of the antidepressant drug minaprine to analogs with nanomolar activities as acetylcholinesterase inhibitors, corticotrophin releasing factor (CRF) receptor antagonists and muscarinic M1 agonists [53], and from these M1 agonists further to 5-HT3 antagonists [55].

Prodrugs and soft drugs

Converting drug candidates with good in vitro properties but insufficient in vivo properties, e.g. poor bioavailability, into prodrugs, is a general strategy in lead optimization. Very first examples have been acetylsalicylic acid (however, in this case producing a completely new mechanism of action, see above) and heroin, the diacetyl derivative of morphine. Monoesters of diacidic angiotensin converting enzyme (ACE) inhibitors, e.g. enalapril (its active form is the free diacid), use the amino acid transporter for active uptake. As prodrugs have been extensively reviewed, only a few examples shall be mentioned here. Some antiviral nucleoside analogs behave as Trojan horses. They are only activated in virus-infected cells by viral kinases, to mononucleotides, that are further phophorylated to trinucleotides by cellular kinases. Due to their chemical structure, biosynthesis of the growing nucleic acid chain of the virus stops after their insertion. The anti-ulcer drug omeprazole has not been developed as a prodrug but it turned out to be a drug with the, most probably, best organ selectivity. In an acid-resistant formulation it passes the stomach, is absorbed in the intestine and is distributed all over the body. In the acid-producing cells of the stomach, and only there, it is activated by an acid-catalyzed rearrangement to irreversibly react and inhibit H+/K+-ATPase, the so-called proton pump (for further details on prodrugs see textbooks of medicinal chemistry, e.g. [25,26,33]).

Soft drugs are active derivatives of inactive drug analogs, e.g. esters of corticosteroid 21-acids, which are topically active but are immediately metabolically degraded to the biologically inactive 21-acids after dermal absorption.

Biological activities of enantiomers the chiral switch

In the past, chiral drugs were developed as racemates or as diastereomeric mixtures, if two or more chiral centers were present. Only about 20 years ago, the pharmacologist Ariëns critisized racemates as compounds "including 50% impurity" [56] to make pharmaceutical industry aware of the problem that a drug and its mirror image might have significantly different biological activities. Indeed, some chiral barbiturates are sedative in their active form, whereas their enantiomers cause convulsions; with some synthetic morphine analogs, the one enantiomer is a strong analgesic, whereas the other one is an antitussive drug; some dihydropyridines are calcium channel blockers in their one enantiomeric form, whereas the mirror image stabilizes the calcium channel in its open form, leading to a compensation of biological effects in the racemate. In the case of ibuprofen, the R(-)-form is metabolically converted to the biologically active S-(+)-form but not in the other direction. Another example is thalidomide: although the different enantiomers are responsible for sedative activity and teratogenic side effects, respectively, a separation would not help due to metabolic interconversion of both enantiomers.

In the last decade, companies have extended the lifetime of their chiral drugs, if originally marketed as a racemate, by a so-called "chiral switch" (e.g. [57]), i.e. by marketing the biologically active enantiomer instead of the racemate. Examples of this strategy are dexfenfluramine (withdrawn 1997), dexibuprofen, dexketoprofen, levofloxacin, levalbuterol, levobupivacaine, esomeprazole, levocetirizine, dexmethylpenidate, and escitalopram [57].

Rescuing poor leads the metabolic switch

Sometimes, leads have such poor properties that neither classical optimization nor a prodrug derivative can help. Nevertheless, such compounds can be "rescued", either by understanding the biochemical mechanisms, by selecting a metabolic precursor, or by selecting an active metabolite of an otherwise inactive or toxic drug. The four examples dopamine, phenacetin, terfenadine, and zanamivir shall illustrate these approaches.

Parkinson's disease results from a lack of dopamine in certain brain areas. The simplest imaginable therapy, a substitution by oral application of dopamine, is impossible due to its poor bioavailability and insufficient blood-brain barrier penetration. L-Dopa, the metabolic precursor in its

biosynthesis, offers a good chance because it is actively transported, in absorption as well as through the blood brain barrier. However, peripheral side effects, like increase of heart rate and blood pressure, and short biological halflife time limit its therapeutic value. Both are compensated by co-application of a polar dopa decarboxylase inhibitor, which acts only in the periphery, and a centrally active monoamine oxidase inhibitor, resulting in a unique success of rational combination therapy. Phenacetin has been used for decades as a mild analgesic and antipyretic principle before liver toxicity and nephrotoxiticity after chronic abuse caused its withdrawal from the market. Its active metabolite paracetamol does not form these toxic metabolites and has replaced phenacetin. Similarly, the non-sedative H1 antagonist terfenadine had to be replaced by its active metabolite fexofenadine, because terfenadine itself is a hERG (human ether-a-go-go-related gene) channel inhibitor. Whereas under normal conditions it is rapidly oxidized to fexofenadine, it becomes extremely toxic if its metabolism is inhibited by co-medication of a CYP3A4 inhibitor, like ketoconazole, erythromycin, grapefruit juice and many other agents [58,59]. Zanamivir, the first neuraminidase inhibitor for the treatment of influenza (see below) [60], is so polar that it can only be applied by inhalation. Inspection of its chemical structure does not offer any reasonable clue to convert it into an orally active drug. However, the chance observation that analogs without the typical glycerol side chain of sialic acid analogs are also biologically active [61,62], led to the development of the orally available drug oseltamivir, which is an ethyl ester prodrug of a lipophilic transition state analog [62]. Although these examples are individual success stories, they demonstrate that poor leads can indeed be converted into valuable drugs.

Screening and high-throughput screening (HTS)

Most drugs result after more or less systematic optimization of lead structures that were discovered by testing the compounds in animals, isolated organs, or in vitro, in enzyme inhibition or receptor binding models. Benzodiazepines, naftifine, cyclosporin A, coumarins as HIV protease inhibitors [63-65], and several non-peptidic antagonists of peptide G protein-coupled receptors, to mention only a few prominent examples, resulted from screening.

Thus, there is no question that screening contributed to the discovery of many valuable leads. However, with automated high-throughput screening, the situation became more difficult. Despite the fact that e.g. nevirapine, delavirdine, efavirenz, bosentan, gefitinib, and sivelestat evolved from lead structures discovered through HTS [66], companies are now aware that the original concept to throw their compound collections, any commercially available compounds, or combinatorial libraries (see next section) on many new biological targets does not deliver to the expected extent. Limited solubility, deposition after dilution with buffer, compound decomposition in the storage solution, as well as unknown concentrations, colored impurities, fluorescence of some compounds, etc., produce legions of false negatives and false positives. In many cases, re-testing does not confirm any primary hits, in other cases, re-testing of analogs that are similar to confirmed hits uncovers their activity, although they were initially found to be inactive. One potential reason for such problems is the promiscuous "activity" of certain compounds at many different targets [67,68]; such compounds cause an agglomeration of the protein, in this manner pretending biological activity.

Another important question arises: is target focus really the best strategy or were whole animal experiments better suited for the search of new leads? There is no way back to animals as screening models but one has to consider that several drugs, e.g. antidepressants and neuroleptics, exert a broad spectrum of different activities; a most prominent example is the atypical neuroleptic drug olanzapine, which binds with nanomolar affinities to more than a dozen different G-protein-coupled receptors [36].

Combinatorial chemistry

Even more disappointing than HTS results with historical compound collections was the success rate of combinatorial libraries, especially in the early years. Huge libraries of ill-defined mixtures of most often lipophilic and too large compounds were tested, without any positive result. Only after introduction of the Lipinski rule of five [13] and other virtual screening techniques people became aware of the importance of certain drug properties, like appropriate molecular weight and balanced lipophilicity. Hann et al. [8,9] gave evidence that the hit rate of libraries generally decreases with an increase

in the number of "over-decorated", i.e. too large and too complex molecules. In addition, they proposed to change strategies in the synthesis of libraries, e.g. to synthesize only hundred R1-modified analogs with constant R2 and R3 groups, hundred R2-modified analogs, etc., instead of a million of analogs with all possible variations of R1, R2 and R3 in a molecule with three different positions of substitution and 100 R variations in each position.

In the meantime, combinatorial chemistry developed into automated parallel synthesis of much smaller libraries of single and pure (or purified) compounds of biological interest. Its main application is nowadays not so much in lead structure search but in lead validation and in the early phases of lead optimization. Schreiber et al. [69,70] described the synthesis of a 2.18 million compounds library of natural product-like compounds but no biological activities have been described for these compounds, so far. Better recommendations for the synthesis combinatorial libraries of natural product analogs have been given by Waldmann et al. [71]. Weber proposed the synthesis of high-diversity libraries, based on multi-component reactions that generate a multitude of different scaffolds [72]. A convincing example of the proper application of combinatorial chemistry in early lead profiling is, e.g. the discovery of nanomolar somatostatin receptor subtype-selective ligands in several libraries, with up to 350,000 members per library [45].

Virtual screening

In classical medicinal chemistry, drug discovery always started from a lead (see sections above). In this approach, the often-quoted ratio of one drug per 10,000 new molecules was a realistic estimate. In our time, with combinatorial chemistry and high-throughput screening, this ratio changed to hundred thousands or even millions test compounds for a new drug. Relatively often, no hits at all are discovered in HTS and the corresponding target is then called a "non-druggable" target. But even in positive cases, not every screening hit can be confirmed and later validated by the synthesis of close analogs and not all validated hits are suited as leads, according to their physicochemical properties [73].

Virtual screening is a toolbox of methods to select appropriate candidates, in order to enrich compound collections and combinatorial libraries with promising candidates [74-81]. As the input of these techniques are only chemical structures and calculated properties of the compounds, virtual screening can also be applied to virtual libraries of almost any size. Most important is a proper pre-processing of the databases, including the removal of duplicates and counterions, defining the right protonation state, e.g. by a set of rules (a problem that still awaits a satisfactory solution), and defining the most prominent tautomer of a compound, or all possible tautomers. Especially for similarity searches, the superposition of molecules, pharmacophore searches and docking, the correct definition of hydrogen bond donor and acceptor properties is of utmost importance (e.g. [82]). The Lipinski rule of five [13] should be applied for the selection of orally bioavailable compounds, whereas neural nets have been trained for the identification of drug-like compounds [74,83-85]. Another virtual screening method has been derived to identify "frequent hitters", i.e. molecules that show up as hits in many different biological assays [86]. Filters for cytotoxicity, toxicity, mutagenicity and cancerogenicity should be considered with suspicion and applied with extreme care; first of all, too many different filters may eliminate too many false positives (e.g. non-toxic molecules considered to be toxic) and second, most of these filters have a poor test set predictivity, coming close to chance prediction.

Feature trees [87,88] are an approach for an extremely fast comparison of molecules; they are especially suited for the evaluation of screening results and the subsequent search in huge virtual libraries. For a more precise comparison and superposition, the program FlexS can be used [89,90]. CATALYST is a program for the generation of pharmacophore hypotheses and 3D database searches [73].

Structure-based ligand design

The large number of protein 3D structures that is available from the Brookhaven Protein Database (22,823 entries; August 01, 2003) [91] enables scientists to perform, in principle, a de novo construction of ligands that fit a certain binding site, in shape and in all other properties [92-95]. Structure-based ligand design started about 25 years ago, with Goodford's design of aromatic dialdehydes, which mimicked 2,3-

diphosphoglycerate as an allosteric regulator of hemoglobin, and of trimethoprim analogs with enhanced affinity to dihydrofolate reductase (DHFR) [96]. However, despite being a major breakthrough in drug research, some principal problems of structure-based ligand design arose already at the very beginning. A perfect ligand is not necessarily a good lead for further development: the dialdehydes could not permeate the erythrocyte membrane and the trimethoprim analogs had lost their selectivity for bacterial DHFRs. Several other early attempts ended in failure, due to lack of bioavailability, too high lipophilicity, or insufficient biological halflife time.

The very first drug, which resulted from structure-based design, was introduced into therapy at about the same time. Captopril was derived from a low-affinity lead structure, which was modeled from the 3D-structure of an inhibitor complex of the related enzyme carboxypeptidase [97]. Other drugs followed, e.g. dorzolamide [98], and the HIV protease inhibitors nelfinavir and amprenavir [46]; many more are in clinical development. Being aware about the important other properties of a development candidate, structure-based design is now a most important technique in cases, where the target 3D structure is known or accessible.

Many more 3D structures of proteins and protein-ligand complexes will become available in the near future, due to high-throughput techniques in protein crystallization and crystallography [99]. Several structural genomics initiatives aim to concentrate on the 3D structure determination of proteins with supposed new folding patterns. Once the major part of all protein folds will be known, homology modeling and molecular replacement in crystallography will gain further importance. Some problems in the application of X-ray crystallographic data in drug design have been discussed by Davis et al. [100].

Computer-aided ligand design

Molecular modeling [101,102] started about 25 years ago, with the presentation and real time rotation (!) of a molecule in front of a computer screen. Within short time it developed to a highly valuable tool in drug design, especially supporting the medicinal chemist to establish and evaluate working hypotheses on structure-activity relationships. A very first computer-assisted approach to generate active molecules de novo, was the program CAVEAT [103], which replaces a peptide loop by a (rigid) scaffold that is capable to accommodate the relevant amino acid side chains in exactly the same 3D orientation as the peptide lead. In this manner, a peptidomimetic is created in one step; the conversion of peptidic integrin ligands to benzodiazepines [41,42] might be considered a successful application of this concept.

Goodford's computer program GRID [104-106] inspects the surface of a protein, especially its binding site, with different chemical probes, to search for "hot spots" where a certain functionality of a ligand should favorably interact. The most impressive application of structure-based and computer-aided drug design resulted from the application of GRID to the viral enzyme neuraminidase: von Itzstein inspected its 3D structure and discovered a pocket, where a positively charged substituent at a low-affinity lead structure should enhance biological activity. This was indeed the case: introduction of a guanidinium group into this lead increased affinity by about 4 orders of magnitude, leading to the influenza drug zanamivir [60]. An alternative to GRID is the program IsoStar [107], which extracts a statistics of nonbonded intermolecular interactions from the Cambridge Crystallographic Database [108]. SuperStar [109-111] is an extension of IsoStar; contour maps are generated from the individual positions of the interacting groups.

Besides some other, more restricted prototypes, a first computer program DOCK was developed by Kuntz for the geometric docking of ligands into a binding site [112]. Further progress resulted from the program LUDI [113,114], which defined interaction sites and used a scoring function [115] to evaluate the docking results. Programs for a flexible docking of ligands into a rigid binding site are e.g. DOCK 4.0 [116], GOLD [117], FlexX [118,119], and the public domain program AutoDock [120,121]; the FlexX modifications FlexE [122] and FlexPharm [123] allow a flexible ligand docking into an ensemble of different binding site conformations and the definition of pharmacophore constraints, respectively. About two dozens different docking programs and several success stories of computer-assisted drug design were reviewed by Schneider and Böhm [78]. Affinity estimations of ligands in different binding

geometries are still a major problem, which is e.g. illustrated by a recent comparison of the performance of a major number of different scoring functions [124]. By careful inspection of protein 3D structures, Nissink et al. collected a standard set of 305 validated ligand-protein complexes, with protonation states assigned by manual inspection [125]; this set is recommended for further scoring function evaluations.

Fragment-based ligand design

The chance of a ligand to bind to a protein depends on its complexity [8]. Smaller ligands have more possibilities to be accommodated, which was considered in the first small ligand library of the program LUDI [113,114], as well as in the MCSS (multiple copy simultaneous search) docking program [126], which uses functional groups and small molecules to search for an ensemble of favorable locations within the binding site. Needle screening [127,128] is a strategy to start from small ligands that have optimal properties (e.g. high affinity and selectivity) and to extend these molecules to larger ligands. Already forty years ago it has been observed that a transition state inhibitor has a much higher affinity than its fragments [129]. An even more pronounced effect is observed for the binding of biotin fragments to avidin; whereas the fragments have only micromolar affinities to avidin, biotin itself binds with femtomolar affinity [130]. Page and Jencks explained this huge increase in affinity by the so-called anchor principle [131,132]: on binding, any molecule looses its degrees of translational and rotational freedom [133]; as this entropic contribution is more or less constant for all molecules, the binding of fragments is less favored than the binding of one ligand. The anchor principle has been confirmed by several other investigations (e.g. [133,134]) and it has recently been used in the rational design of a nanomolar enzyme inhibitor, starting from two low-affinity natural products that bind to adjacent sites of the protein [135].

Surprisingly, the concept of combining two (or more) low-affinity ligands to a high-affinity ligand has not been systematically used, until Fesik developed the SAR by NMR strategy [136-138]. This experimental method searches for relatively small, low-affinity ligands of small proteins. Whenever such a ligand is discovered, the corresponding binding site is saturated with this ligand and other low-affinity ligands are searched that bind at adjacent sites. In a last step, a linker combines both molecules to a nanomolar ligand [136-138]. Since NMR techniques are superior to other approaches in the detection of low-affinity ligands, the SHAPES method [139,140] has been developed for the search of new leads and their subsequent optimization, as well as some other NMR-based techniques [141-145].

Electron density maps from X-ray structure analyses of protein crystals, soaked with different solvents, might also be used as a tool in lead discovery [146-152]. The CrystaLEAD method [153] monitors changes in the electron density maps of crystals that were soaked with large libraries of potential ligands; however, this approach has not yet been fully exploited. On the other hand, soaking of protein crystals with a mixture of only a few small ligands that differ in size and shape, in combination with high-throughput crystallography, seems to be a very promising new approach in lead discovery [99,154].

Combinatorial ligand design

The concept of fragment-based ligand design has been extended to combinatorial techniques [155], where a multitude of ligands is tested in the search for new leads. An elegant screening method uses microarrays of low-molecular weight ligands [156]; up to 10,000 compounds can be tagged to a gold-coated glass surface via an anchor molecule that carries a reactive group. Binding of any protein to the immobilized ligand is detected by surface plasmon resonance; the advantage of this approach is its independence on the development of a specific screening method for a new protein; certain problems may arise from the restricted mobility and accessibility of the ligands. The dynamic assembly of ligands [157-160] generates ligands from fragments that are capable to reversibly react with each other in the presence of a protein. Ligands that fit the binding site are preferentially formed and afterwards trapped by a reaction that freezes the equilibrium (e.g. hydrogenation of Schiff bases); the application of this principle has been illustrated by the generation of carbonic anhydrase [157] and neuraminidase inhibitors [160]. The discovery of low-affinity ligands can also be achieved by introducing a cysteine residue into the biological target, close to the binding site; disulfide forma-

tion stabilizes the binding of sulfhydryl-containing low-affinity ligands [161,162]. Some other approaches for the combinatorial design of new leads have recently been described [163,164].

An elegant method for the formation of ligands from different fragments uses spontaneous chemical reactions ("click chemistry"), which are significantly accelerated if the reacting groups of two molecules come close together in the binding site of a protein; femtomolar acetylcholinesterase (AChE) inhibitors resulted from a mixture of fragments that were capable to react with each other in an irreversible manner [165]. A promising stochastic principle for the generation of new leads is the so-called "random chemistry" approach; molecules are irradiated in the presence of a matrix (e.g. a solvent), to form analogs with unprecedented chemical structures and biological activities; new thymidine kinase substrates and inhibitors have been generated in this manner [166].

In addition to these experimental techniques, there are several computer-assisted techniques for the combinatorial combination of fragments to new leads. A first step in this direction was a computational algorithm to design ligands that are available from a single-step chemical reaction [167]. The design of combinatorial libraries with a high percentage of drug-like compounds can be achieved with the program CombiGen [168]. The program uses privileged and/or user-defined fragments and reassembles them, with or without minor chemical modifications, to new structures; subsequently, virtual screening procedures eliminate molecules with undesired properties. TOPAS [169,170] is a program which dissects lead structures into fragments and assembles new molecules by re-combining a chemically similar scaffold with similar fragments; split and cleavage of the molecules follow chemical reactions that are defined in a RECAP-like procedure [171]. In this manner, a "scaffold hopping" [172] is achieved, leading into new chemistry. In principle, a docking program like FlexX [118,119], which performs an incremental construction of a ligand within the binding site, could arrive at comparable results, if a multitude of different building blocks is offered to the program, instead of the original building blocks; instead of constructing a virtual library of millions of potential candidates, only interesting partial solutions would be generated and proceeded to the next steps. However, more reliable scoring functions [124] are needed to achieve this task.

Summary and Conclusions

If one considers the broad range of approaches to arrive at new leads, it is surprising that lead search indeed poses a problem. However, traditional sources, like plant products, microbial metabolites, endogenous neurotransmitters and hormones, are to some extent "exhausted". High-throughput screening (HTS) and combinatorial chemistry did not deliver to the expected extent. Virtual screening and fragment-based approaches have just started but they seem to be the most powerful techniques for the near future [81]; compound collections and virtual libraries can be enriched with promising candidates which can be tested with greater care than usually applied in routine HTS runs. In the very end, the integration of protein crystallography, NMR techniques, and virtual screening will "significantly enhance the pace of the discovery process and the quality of compounds selected for further development" [173]. After several success stories of structure-based design of enzyme inhibitors, the time has come to successfully apply this technique also to GPCR homology models [174,175].

In their search for new leads, as well as in lead optimization, medicinal chemists always followed the similarity principle, that similar compounds should exert similar biological activities. Despite many exceptions to this general experience [176,177], drug research focuses now very often on target families. The term "chemogenomics" has been coined for the investigation of certain compound classes in target families, like the G protein-coupled receptors (GPCR), the serine proteases, kinases, etc. [178-181]. On the other hand, it is tempting to speculate whether drug candidates can also be found in regions of the chemical universe which are, so far, not populated by drugs [182]. Considering the failure of early combinatorial chemistry, driven by chemical accessibility instead of drug-like character of the products, it still seems to be more rewarding to search in areas that are already known to deliver drug candidates; it might well be that drug space is not evenly distributed within chemical space.

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