Drug Discovery & Development

How new bioactive molecules are discovered
How candidates are developed into drugs
How drugs find their way to the market
Goals:

• Comprehensive picture of the Drug Discovery & Development process.

• Get a «feeling» for what is a good molecule & what molecules are good for.

• Develop an understanding on how it works & what processes are involved.
<table>
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<th>Uhrzeit</th>
<th>Raum</th>
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<tbody>
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<td>SR 2</td>
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<td>SR 1</td>
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<td><strong>SR 2</strong></td>
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<td>SR 4</td>
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The Beginnings
Antikamnia Calendars and the Birth of Tylenol

These mischievous little skeletons helped lead us to one of today’s most successful pain relievers.

The Antikamnia company marketed an analgesic (pain-relieving) powder to pharmacists and druggists of the late 19th and early 20th centuries using these rather morbid calendars. The name of the company actually means “opposed to pain”.

Antikamnia Chemical Company was later shut down after failing to disclose the active ingredient of their pain-relieving products: Acetanilide. Not only was it illegal (as it is now) to fail to label drugs correctly, but acetanilide was known to impair red blood cells’ ability to release oxygen to tissues. That’s not the kind of drug you want on the market, obviously.

But Anitkamnia was an effective pain reliever, even if you’d go blue after taking it. One thing many people don’t realize about pharmaceutical chemicals is that they are metabolized and modified by human biochemistry. For many of them, the compound in the pill is useless, and they require breakdown or modification to become active. It wasn’t until nearly half a century later that Nobel Prize-winning biochemist Julius Axelrod discovered that the primary metabolic product of acetanilide is a compound called paracetamol.
What was the problem?


Acetanilid (Antifebrin) analgesic, antipyretic

metabolized

Phenylhydroxylamine Toxic via haemoglobin mod.

active

Paracetamol
When Bayer wanted to mimic paracetamol they had access to large quantities of a precursor which would give phenacetin.

Although phenacetin is mainly metabolized to paracetamol (active form) a side reactions lead to toxic metabolites similar to phenylhydroxylamine.
<table>
<thead>
<tr>
<th>Year</th>
<th>Drug</th>
<th>Category</th>
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<tbody>
<tr>
<td>1875</td>
<td>Salicylsäure</td>
<td>Entzündungshemmer</td>
</tr>
<tr>
<td>1884</td>
<td>Cocain</td>
<td>Stimulans, Lokalanästhetikum</td>
</tr>
<tr>
<td>1888</td>
<td>Phenacetin</td>
<td>Analgetikum und Antipyretikum</td>
</tr>
<tr>
<td>1889</td>
<td>Acetylsalicylsäure</td>
<td>Analgetikum und Antipyretikum</td>
</tr>
<tr>
<td>1903</td>
<td>Barbiturate</td>
<td>Schlafmittel</td>
</tr>
<tr>
<td>1909</td>
<td>Arsphenamin</td>
<td>Syphilismittel</td>
</tr>
<tr>
<td>1921</td>
<td>Procain</td>
<td>Lokalanästhetikum</td>
</tr>
<tr>
<td>1922</td>
<td>Insulin</td>
<td>Antidiabetikum</td>
</tr>
<tr>
<td>1928</td>
<td>Estron</td>
<td>weibliches Sexualhormon</td>
</tr>
<tr>
<td>1928</td>
<td>Penicillin</td>
<td>Antibiotikum</td>
</tr>
<tr>
<td>1935</td>
<td>Sulfachrysoidin</td>
<td>Bakteriostatikum</td>
</tr>
<tr>
<td>1944</td>
<td>Streptomycin</td>
<td>Antibiotikum</td>
</tr>
<tr>
<td>1945</td>
<td>Chloroquin</td>
<td>Malariamittel</td>
</tr>
<tr>
<td>1952</td>
<td>Chlorpromazin</td>
<td>Neuroleptikum</td>
</tr>
<tr>
<td>1956</td>
<td>Tolbutamid</td>
<td>orales Antidiabetikum</td>
</tr>
<tr>
<td>1960</td>
<td>Chlordiazepoxid</td>
<td>Tranquillizer</td>
</tr>
<tr>
<td>1962</td>
<td>Verapamil</td>
<td>Calciumkanalblocker</td>
</tr>
<tr>
<td>1963</td>
<td>Propranolol</td>
<td>Blutdrucksenker (Betablocker)</td>
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<tr>
<td>1964</td>
<td>Furosemid</td>
<td>Diuretikum</td>
</tr>
<tr>
<td>1971</td>
<td>L-Dopa</td>
<td>Parkinsonmittel</td>
</tr>
<tr>
<td>1975</td>
<td>Nifedipin</td>
<td>Calciumkanalblocker</td>
</tr>
<tr>
<td>1976</td>
<td>Cimetidin</td>
<td>Ulcusmittel (H₂-Blocker)</td>
</tr>
<tr>
<td>1981</td>
<td>Captopril</td>
<td>Blutdrucksenker (ACE-Hemmer)</td>
</tr>
<tr>
<td>1981</td>
<td>Ranitidin</td>
<td>Ulcusmittel (H₂-Blocker)</td>
</tr>
<tr>
<td>1983</td>
<td>Cyclosporin A</td>
<td>Immunsuppressivum</td>
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<tr>
<td>1984</td>
<td>Enalapril</td>
<td>Blutdrucksenker (ACE-Hemmer)</td>
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Figure 1.3 - Some major therapeutic innovations by decade. (Reprinted with permission from “Biopharmaceutical Industry Contributions to State and US Economics.” Available at www.milkeninstitute.org/pdf/biopharma_report.pdf, Milken Institute.)
Success rates

1.000.000 compounds

3 - 5

10.000 chemical derivatives

100

Research

Development

10

3

1 new drug

High-Throughput Screening (HTS)
Lead Structures
Chemical Optimisation
Pharmacology
Preclinical Development
Clinical Development
Product Launch
Success rates per development phase

- Pre-Clinical: 60%
- Clinical I: 40%
- Clinical II: 60%
- Clinical III: 85%
- Registration: 85%
- Launch: 100%

Value/costs: 1 billion USD

Time: 14 years
Success rates per development phase

Conventional discovery and development approaches represent a heuristic. In most, but not all, discovery programmes, the processes begin with selection of an interesting protein target. Screening, or hit-to-lead, and then lead optimisation, processes match a likely lead candidate to the protein target; pharmacokinetics, toxicological features and basic efficacy are examined, usually in proxy or simplified systems, and then the efficacy and safety risks are evaluated somewhat serially by seeing whether these risks manifest in people, in the clinic. During risk evaluation in the clinic, the entire spend on the development programme to that point hangs on the possibility that the trial, usually expensive in itself, will not manifest an important efficacy or safety risk.

The success rate of this heuristic approach is very low. For example, the average probability that a candidate emerging from lead optimisation will not make it to be a drug is above 99.8%². Building

Hence, a very great deal turns on whether one can in fact predict accurately what the efficacy, safety and deliverability issues of a candidate molecule are – before undertaking expensive test-

Prediction v Attrition
DDW 2008, Fall
Number of annual approvals & costs

New drug approvals (dots), represented on the left vertical axis, and pharmaceutical R&D expenditures (shaded area), represented on the right vertical axis, in the United States from 1963 to 2008. R&D expenditures are presented in terms of constant 2008 dollar value. The trend line is a 3-year moving average. The source of drug approval data is the Tufts Center for the Study of Drug Development (CSDD). The source of R&D expenditure data is the Pharmaceutical Research and Manufacturers of America; Industry Profile 2009; conversion of actual expenses to constant dollars was performed by Tufts CSDD.
Costs & time for bringing a drug to the market

Number of patients and costs for clinical trials

### Table 3. Estimated clinical trial expenditures for recent obesity drugs

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Est. Costs (SMM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lorcaserin (Lorc)</td>
<td>Qnexa (Qnexa)</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Phase I</td>
<td>104</td>
</tr>
<tr>
<td>Phase II</td>
<td>1,065</td>
</tr>
<tr>
<td>Phase III</td>
<td>7,794</td>
</tr>
<tr>
<td>Total</td>
<td>8,963</td>
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</tbody>
</table>

### Table 6. Estimated clinical trial expenditures for recent drugs in selected rare diseases

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Est. Costs (SMM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lorcaserin (Soliris)</td>
<td>Gattex</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Phase I</td>
<td>11</td>
</tr>
<tr>
<td>Phase II</td>
<td>11</td>
</tr>
<tr>
<td>Phase III</td>
<td>184</td>
</tr>
<tr>
<td>Total</td>
<td>206</td>
</tr>
</tbody>
</table>

Source: Arena, Orexigen, Vivus SEC filings; Cutting Edge Information

Source: Alexion, NPS SEC filings; NIH; Cutting Edge Information
Summary Process characteristics

- Process is highly fragmented (specialized)
- Standardized (regulatory & heuristic reasons)
- Chases a rare event – success (complexity)
- Unidirectional – failure to progress is failure (no going back)

Let’s look at some significant benchmark figures...
What goes wrong, when things go wrong....
(case study from Astra Zeneca – Nat. Rev. DD 2014, 13, 420).

Box 1 | Case study 1: the mGluR2 modulator AZD8529 for schizophrenia

AZD8529 is a positive allosteric modulator (PAM) of the presynaptic autoreceptor metabotropic glutamate receptor 2 (mGluR2). The rationale for mGluR2 as a target for schizophrenia was based on the hypothesis that failure of the cortical glutamatergic drive results in disinhibition of subcortical dopamine function, which in turn leads to psychosis. The hypothesis predicts that normalization of glutamatergic function should have antipsychotic effects, with stimulation of mGluR2 proposed to provide such normalization.

Before initiating a Phase II study, Lilly had disclosed that its dual mGluR2 and mGluR3 agonist demonstrated a statistically significant improvement in patients with acute psychotic schizophrenia, although the results of a subsequent dose-finding study were negative. AZD8529 was active in seven preclinical models that were used to predict antipsychotic activity as well as in two preclinical models of anxiety. Effective plasma exposures for these models covered a broad range, from 2 nM to over 1,000 nM. Toxicology studies revealed testicular lesions in the dog models and cataracts in the rat models, which limited the maximum dose that could be given to humans. The dose for the Phase II study, 40 mg given every other day, was chosen based on the plasma exposure associated with the ‘collective activity’ in animal models and the need to provide a sufficient margin bearing in mind the toxicology findings. Limited cerebrospinal fluid (CSF) sampling conducted during the multiple-dose study indicated that free drug exposure in the CSF was roughly 50% of the free plasma exposure in humans. Efforts to develop a positron emission tomography (PET) ligand had been unsuccessful and there was no direct way to assess target engagement.

The Phase II study was a 4-week placebo-controlled study in 152 patients with symptomatic schizophrenia who were diagnosed and assessed using standard methods. Patients were randomized in a 2:2:1 ratio to receive AZD8529, placebo or the marketed antipsychotic risperidone. Risperidone had a robust effect on positive and negative symptoms relative to placebo, but no effect was observed with AZD8529. Following the negative Phase II study, further development in schizophrenia was stopped. Lilly also ceased development of its dual mGluR2 and mGluR3 agonist following the failure of two Phase III trials (see the 29 August 2012 press release on the Lilly website).
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Before initiating a Phase II study, Lilly had disclosed that its dual mGluR2 and mGluR3 agonist demonstrated a statistically significant improvement in patients with acute psychotic schizophrenia\(^6^3\), although the results of a subsequent dose-finding study were negative. AZD8529 was active in seven preclinical models that were used to predict antipsychotic activity as well as in two preclinical models of anxiety. Effective plasma exposures for these models covered a broad range, from 2 nM to over 1,000 nM. Toxicology studies revealed testicular lesions in the dog models and cataracts in the rat models, which limited the maximum dose that could be given to humans. The dose for the Phase II study, 40 mg given every other day, was chosen based on the plasma exposure associated with the ‘collective activity’ in animal models and the need to provide a sufficient margin bearing in mind the toxicology findings. Limited cerebrospinal fluid (CSF) sampling conducted during the multiple-dose study indicated that free drug exposure in the CSF was roughly 50% of the free plasma exposure in humans. Efforts to develop a positron emission tomography (PET) ligand had been unsuccessful and there was no direct way to assess target engagement.

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What goes wrong, when things go wrong....
(case study from Astra Zeneca – Nat. Rev. DD 2014, 13, 420).

Phase 0 – II:

Phase III/Reg:

Figure 4 Root-cause analysis for 359 phase 3 and 95 NDA/BLA suspended programs. A program was designated as ‘suspended’ when conclusive evidence had been gathered regarding a company’s plans to discontinue development or communications with regulators were not reinitiated for several years.

Nat. Biot. 2014, 32 (1), 40
No 1 reason for failure - SAFETY
No 2 reason for failure - Efficacy
Lessons learned

Additional problem: «truth seeking vs progression-seeking behavior»

Right target
- Strong link between target and disease
- Differentiated efficacy
- Available and predictive biomarkers

Right tissue
- Adequate bioavailability and tissue exposure
- Definition of PD biomarkers
- Clear understanding of preclinical and clinical PK/PD
- Understanding of drug–drug interactions

Right safety
- Differentiated and clear safety margins
- Understanding of secondary pharmacology risk
- Understanding of reactive metabolites, genotoxicity, drug–drug interactions
- Understanding of target liability

Right patients
- Identification of the most responsive patient population
- Definition of risk–benefit for given population

Right commercial potential
- Differentiated value proposition versus future standard of care
- Focus on market access, payer and provider
- Personalized health-care strategy, including diagnostic and biomarkers

Figure 5 | The 5R framework. Summary of the key features of the five-dimensional framework that can be used to describe a drug discovery and development project. PK/PD, pharmacokinetics/pharmacodynamics.
Applying the ‘5R’s

This programme failed before we implemented the ‘5R’ strategy, but can be analysed retrospectively in this framework. Before the initiation of the Phase II study, we only had low confidence that mGluR2 represented the ‘right target’ for the treatment of schizophrenia, on the basis of the well-documented limitations of preclinical models of efficacy in this indication, particularly with novel mechanisms\textsuperscript{64}. Moreover, there was no clear understanding of how the underlying biology was linked to the disease. Similarly, there was low confidence in the ability to select the ‘right patients’ for this study because no information was available to identify the most appropriate patients for this study beyond the phenomenological diagnosis of schizophrenia.

However, the unmet medical need drove high confidence in the ‘right commercial potential’ dimension. The low confidence in the ‘right target’ and the ‘right patient’ dimensions focused attention on the remaining two ‘R’s; the ‘right tissue’ and the ‘right safety’. Confidence in the ‘right tissue’ dimension was low owing to the variable exposure observed as well as our failure to develop a PET ligand or other biomarker by which to measure target engagement. The toxicity, a side effect that was unacceptable for this indication, also raised concerns with regard to the ‘right safety’ and consequently limited dose selection in the clinic and the ability to test exposures that were likely to provide adequate target engagement.

Taken together, the inability to directly measure drug exposure at the target tissue at the appropriately predicted doses, coupled with clear gaps in our knowledge of the target and of patient selection, made it highly unlikely that this project would be successful. Applying the 5R framework would have identified many of these issues and gaps early on, and if they had not been addressed and solved by the team they would have prevented the programme from progressing into expensive proof-of-concept studies.

The case: Despite technology improvements – it gets harder & harder:
Unique situation of Pharmaceutical products:

1. The «better than the beatles» problem

Unlike in most other industries, previous developments generate a back-catalogue of approved medication. Any new one needs to have a clear benefit i.e. any new medicine must be better than the standard-of-care available.

2. The «cautious regulator» problem

Very similar to the above, the advance in standards also leads to an ever tightening environment of regulations which slows down the process, makes additional testing necessary and prolonges the cycle time.

3. The «more money will solve it» attitude

The tendency to react to increased challenges from 1 & 2 by increasing resources increases also the costs. Increasing resources however, does not necessarily lead to more efficient processes.

4. The «numbers instead of science» tendency

In line with 3 there is the tendency to try and compensate the low success rates by increasing the number of projects (i.e. If it gets harder, try to run faster). However, each project is a scientific endeavour and needs to be treated like it, not like a «factory for manufacturing standard parts » where effort/speed = output.
Idialized Drug Discovery
& development flow chart

Disease area
basic research;
Medical disease
understanding

Assay strategy
Assay development
Assay validation

Med. Chem. optimization

Process development,
Disease marker,
endpoint definition

Random Models
Preclinical in-vivo
profiling

Screening

Lead Selection
based on «lead criteria»

Hit Selection
Hit to «Lead»
early chemistry

Animal Models
Preclinical in-vivo
profiling

Hit confirmation,
Hit validation
«counter screening»

IND
Investigational New
Drug application

Clinical Phase I

NDA
NEW DRUG
APPLICATION

Clinical Phase III

Clinical Phase II

Compound repository
Archive

Clinical Phase III
The beginnings – serendipidty & natural molecules

Figure 1.3 Some major therapeutic innovations by decade. (Reprinted with permission from “Biopharma-ceutical Industry Contributions to State and US Economics.” Available at www.milkeninstitute.org/pdf/biopharma_report.pdf, Milken Institute.)
1. **Antipyretic/analgesic:** Aspirin, Paracetamol,
   Local anaesthetic: Cocain (first applied by Carl Koller
   friend of Sigumnd Freud)

2. **Antiparasitic: Quinine**
   Malaria was endemic in southern parts of Europe
   since 17th century ground bark of cinchona was used
   In 1820 Pelletier and Caventou isolated for the first time Quinine

« We know how advantageous it is in the treatment of disease
to be certain of the precise dose of all active remedies; this advantage especially applies
to the present case, because the quantity of the alkalis contained in the cinchonas bark
varies, according to the nature and quality of the bark. it is often also very desirable to
administer this medicine in a small volume, and in an agreeable form.
Patients often die of malignant fevers because they cannot swallow the necessary
quantity of the bark in powder. Some throw it up after having taken it. «

From «Formulaire pour la Preparation et l’Emploi de Plusiers Nouvais Mèdicaments»,
the first compendium describing pure chemicals instead of plant mixtures.
2: **Antiparasitic:** Salvasarn - The first safe and effective medicine against syphilis. The active structure was based on earlier work with dyes.

3. **Barbiturates:** Hypnotics, anesthetics, anticonvulsant, anxiolytic

Joseph von Mering studied earlier hypnotics and was convinced that a carbon atom (3 or 4 substituents, containing two ethyl-groups) would be the central active feature of hypnotics. He worked with Emil Fischer and synthesized diethylacetylurea – and it was found to be have the desired activity.

He then prepared 5,5-dethylbarbituric acid. Because Emil Fisher doubted the synthesis of his student, he re-synthesized the compound. It was found much more active therefore, Fished called it «Veronal» The compound was later marketed by Bayer as «Veronal»

**Barbituric acid, first synthesized 1864 by Adolf von Bayer**
4. **Corticosteroids**: looking for molecules in a new way..

19th century, a condition called Addison’s disease was first described. Symptoms: weakness, abdominal pain, progression to coma. Cause: insufficient adrenal cortex hormons.

Originally, adrenal glands were used to extract such hormons.

1941: American intelligence agents picked up a rumor that Germany was purchasing high quantities of adrenal glands from slaughterhouses in Argentina, supposedly for the production of extracts to enable Luftwaffe pilots to withstand stress of flights at high altitudes. -> a collaboration with pharmaceutical industry (Merck) was started to synthesize the corticosteroids.

1948: 17-hydroxy-11-dehydrocorticosterone was synthesized.

Observations that woman suffering from arthritis did generally experience less pain during pregnancy led to tests with this compound in arthritis. Merk commercialized the compound as Cortison -> within 5 years -> 50 indications.

1950 Nobel price of Medicine for all involved (Kendall, Reichenstein, Hench).
Rational targeting of disease – a man ahead of his time
Rational drug discovery is based on the following reductionistic hypothesis: In the network of protein interactions and signal-transductions it is possible to identify individual proteins (genes) which are disease causing. It is further assumed that a specific inhibition/activation of the function of such proteins/genes is possible using molecules (drugs). The selected proteins are called target.
The classics era (1950s-1990s)
phenotypic screening/«body own molecules» as example
Discovery of Rapamycin

J. Antibiot. 1975, 28, 721

RAPAMYCIN (AY-22,989), A NEW ANTIFUNGAL ANTIBIOTIC
(Received for publication June 17, 1975)

A streptomycete was isolated from an Easter Island soil sample and found to inhibit Candida albicans, Microsporum gypseum and Trichophyton granulosum. The antibiotic-producing microorganism was characterized and identified as Streptomyces hygroscopicus. The antifungal principle was extracted with organic solvent from the mycelium, isolated in crystalline form and named rapamycin. Rapamycin is mainly active against Candida albicans; minimum inhibitory concentration against ten strains ranged from 0.02 to 0.2 μg/ml. Its apparent activity against Microsporum gypseum and Trichophyton granulosum

Sirolimus, Rapamune (Pfizer 1999)
Discovery in the «classical» area – phenotypic screening and mimicking

Identification of the Rapamycin-Producing Streptomycete

Streptomycete strain AY B-994 was isolated from a soil sample collected in Easter Island (Rapa Nui): the soil was diluted in distilled water and the resulting suspensions were plated on yeast-starch agar according to the double-layer technique of Porter et al.\(^1\). After one week of incubation at 28°C the streptomycete colonies were purified by repeated streaking and the pure strains grown separately on yeast-starch agar plates to yield confluent growth. After 4~10 days of incubation, discs (7 mm in diameter) were cut and transferred onto the surface of plates of Bacto-Blood Agar Base (Difco Laboratories, Detroit, Mich.) inoculated with test bacteria and SABOURAUD dextrose agar inoculated with test yeast and dermatophytes. The

• Standard approach was to collect soil/martitime samples from different locations (often collected by researcher on their holidays) for isolation of natural products & testing

• No target protein required; initially applied predominantly for infectious diseases

• Alternative approach: starting from known molecules with known activities
Guanosin

Acyclovir

Histamine

Immepip, selective Histamine H3R agonist

Estradiol

Mestranol (contraceptive)
Case study - beta-blockers

Adrenergic receptors in cardiac tissue (pacemakers)
Other functions: general sympathetic nervous system:
Flight-and-fight syndrome, blood-flow regulation, fat-metabolism.

Adrenalin

Biosynthesis of Adrenalin

Tyrosin → L-Dopa → Dopamin → Adrenalin
ADRENALINE ANTAGONISTS

The first drug to antagonise the effects of adrenaline on the heart was serendipitously discovered by Irwin Slater of Eli Lilly in Indianapolis while testing analogues of isoprenaline as potential long-acting bronchodilators. These were screened for their ability to relax tracheal strips that had been contracted by exposure to pilocarpine in order to simulate the bronchoconstriction of asthma. Between test runs, the strips were treated with adrenaline to ensure they were still responsive. However, those that had been exposed to dichloroisoprenaline did not relax when the adrenaline was added. This antagonism of adrenaline by dichloroisoprenaline was reported by Slater at a scientific meeting in 1957. Neil Moran, at Emory University in Atlanta, then requested samples of the new drug to investigate its effects on the heart. He found that while dichloroisoprenaline antagonised the changes in heart rate and muscle tension produced by adrenaline, it still mimicked the activity of the hormone to some extent.

Moran’s report on the cardiac effects of dichloroisoprenaline interested James Black, who had been invited to join ICI Pharmaceuticals Division (now incorporated in AstraZeneca) at Alderley Park in Cheshire after seeking a grant from the company to further his investigations into coronary artery disease. Black believed that an alternative way of treating angina would be to find a drug that reduced the need of the heart for oxygen rather than merely increasing its supply through the use of vasodilators such as the nitrates. He realised that the heart rate was a major factor influencing the demand of the heart for oxygen, so had been seeking a drug to protect the hearts of patients with coronary disease against the effects of adrenaline and noradrenaline. On reading Moran’s paper, he realised it should be possible to find an analogue of dichloroisoprenaline devoid of intrinsic action when bound to the receptors in the heart.

From: Drug Discovery: A History
By Walter Sneader

Adrenalin

Pronethalol (Alderin)
1st beta-blocker drug marketed 1963 -> 30 patient clinical trial

Cancerogenicity is discovered

Propanolol 1965, Inderal
Practolol, 1970 Eraldin

Atenolol, 1976 Tenormin

Toxic (see paracetamol vs phenacetin)

General motif of beta-blockers

\[ X = O-\text{CH2} \text{ or } () \]
\[ Y- \text{ is meta or para} \]
Case study  - Antihistaminergica

Wide variety of activities. Among others, control of acid secretion in the stomach. -> Ulcers of the intestine are highly prevalent in the population. Historical treatment: surgery

Histamine

Guanylhistamine (partial agonist)  Still partial agonist  Higher potency (still partial agonist)

Low Bioavailability  Burimamid (first clinical candidate)  Metiamid 5-10x more potent

Thiourea suspected problem

Granulozytopenie  Side effect (low granulozytes)
Case study - Antihistaminergica

Metiamid

Granulozytopenie
Side effect
(low granulozytes)

Thiourea
suspected problem

Cimetidin, 1977 (Tagamet)

More potent

Ranitidine (Zantac 1981)
4-5 x more potent

Much less potent

General motif of H2 antihistamines
Case study - Statines

- In the 1950s & 60s it became apparent that elevated plasma choesterol as a consequence of improved diet are a major risk factor for coronary hear diseases.
- Working hypothesis: search for drugs which reduce cholesterol biosynthesis
- Rate-limiting enzyme in the pathway is 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase -> target

Nat. Rev. DD. 2003, 2, 517
In contrast to late stage intermediates, Hydroxymethylglutarate is well soluble and there is no build-up expected if HMG-CoA is inhibited.

1970s: Japanese group identifies «compactin», a compound with significant HMG-CoA reductase inhibition, from fermentation broth of *Penicillium citrinum*.
Compactin showed cholesterol lowering in animal models: rabbit, dog, monkey. First doubts were casted by the fact that no lowering in rat was seen.

Japanese company Sankyo demonstrates effectiveness of compactin for total LDL and cholesterol lowering in patients with heterozygous familial hypercholesterinaemia.

1978: Researchers at Merck found a related natural product: Lovastatin in fermentation broth from Aspergillus terreus (Lovastatin).

1980: Phase I trial by Merck for Lovastatin. Drug reduces LDL and cholesterol levels dramatically, no obvious adverse reactions.

1980: Sankyo stops clinical trials with compactin

1980: Merck promptly suspends clinical trials

1984: After academic trials in high-risk patients, Merck resumes trials.

1987: Lovastatin is marketed (first in class statin).
Early statins are very similar to compactin. Later developments retain substrate/product motif but rely on common synthetic scaffolds.
The postgenomics era (2000s -)

Targets – Targets – Targets – Molecular modes of action

Figure 1.3  Some major therapeutic innovations by decade. (Reprinted with permission from “Biopharma-ceutical Industry Contributions to State and US Economics.” Available at www.milkeninstitute.org/pdf/ biopharma_report.pdf, Milken Institute.)
Rational Drug Discovery – Targets..Targets..Targets

- Rational drug discovery based on concept of specific interaction of a protein with a target protein – one drug – one target – one disease

Q: What makes a good drug target?

Q: How many targets are there?
Number of genes in organisms of different «complexity»
Lessons from past targets - how many & what works best?

- Over past 150 years, 1500 drugs generated
- Acting on ~ 400 targets
- This collection provides data set of «successful cases»
- How many more are there?
- Human genome: 30,000 genes

Drugbank database: 1,542 drugs

- Removal of drugs with no known target: 225 drugs
  - 1,317 drugs acting on 1,236 protein targets

- Removal of drugs with no human target: 192 drugs
  - 1,092 drugs acting on 1,044 human protein targets

- Removal of non-therapeutic targets: 609 non-therapeutic targets
  - 980 drugs acting on 435 therapeutic effect-mediating targets
  - 103 drugs without known therapeutic targets

<table>
<thead>
<tr>
<th>Drug category</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown mechanism</td>
<td>44</td>
</tr>
<tr>
<td>Direct DNA-interacting</td>
<td>29</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>19</td>
</tr>
<tr>
<td>Amino acid</td>
<td>17</td>
</tr>
<tr>
<td>Dietary supplement</td>
<td>13</td>
</tr>
<tr>
<td>Other</td>
<td>102</td>
</tr>
<tr>
<td>Total</td>
<td>225</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Target category</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>105</td>
</tr>
<tr>
<td>Animals</td>
<td>31</td>
</tr>
<tr>
<td>Viruses</td>
<td>29</td>
</tr>
<tr>
<td>Parasites</td>
<td>15</td>
</tr>
<tr>
<td>Fungi</td>
<td>10</td>
</tr>
<tr>
<td>Euryarchaeota</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>192</td>
</tr>
</tbody>
</table>
Lessons from past targets - success cases – target classes

989 drugs acting on 435 therapeutic effect-mediating targets

Top 10 drug indications
- Antihypertensive agents 108
- Antineoplastic agents 91
- Anti-inflammatory agents 66
- Hypnotics and sedatives 42
- Anti-allergic agents 39
- Anticonvulsants 37
- Anti-arrhythmia agents 37
- Antipsychotic agents 37
- Antidepressants 32
- Analgesics 30

Target class
- Receptors 193
- Enzymes 124
- Transporters 67
- Other 51

Current Drug Target classes
- Receptors 44%
- Enzymes 29%
- Transporters 12%
- Other 15%
### Breakdown into protein classes

<table>
<thead>
<tr>
<th>Target class</th>
<th>Number of proteins</th>
<th>Most common therapeutic actions</th>
<th>Number of drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G protein-coupled receptors</td>
<td>82</td>
<td>Antihypertensive, anti-allergic</td>
<td>357</td>
</tr>
<tr>
<td>Ligand-gated ion channels</td>
<td>39</td>
<td>Hypnotic and sedative, anticonvulsant</td>
<td>84</td>
</tr>
<tr>
<td>Receptor tyrosine kinases</td>
<td>22</td>
<td>Antineoplastic, vasodilator</td>
<td>22</td>
</tr>
<tr>
<td>Immunoglobulin-like receptors</td>
<td>21</td>
<td>Immunomodulatory, antineoplastic</td>
<td>28</td>
</tr>
<tr>
<td>Other receptors</td>
<td>12</td>
<td>Immunomodulatory, platelet aggregation</td>
<td>11</td>
</tr>
<tr>
<td>Nuclear receptors</td>
<td>17</td>
<td>Antineoplastic, hormone replacement</td>
<td>76</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC 1 Oxidoreductases</td>
<td>22(11)</td>
<td>Anti-inflammatory, antineoplastic</td>
<td>85</td>
</tr>
<tr>
<td>EC 2 Transferases</td>
<td>21(2)</td>
<td>Antineoplastic, bisphosphonate</td>
<td>33</td>
</tr>
<tr>
<td>EC 3 Hydrolases</td>
<td>43(9)</td>
<td>Antihypertensive, vasodilator</td>
<td>96</td>
</tr>
<tr>
<td>EC 4 Lyases</td>
<td>3(2)</td>
<td>Antihypertensive, diuretic</td>
<td>11</td>
</tr>
<tr>
<td>EC 5 Isomerases</td>
<td>5(0)</td>
<td>Antineoplastic, immunosuppressive</td>
<td>14</td>
</tr>
<tr>
<td>EC 6 Ligases</td>
<td>1(1)</td>
<td>Antineoplastic, antifibrinolytic</td>
<td>4</td>
</tr>
<tr>
<td>Multiple EC groups</td>
<td>2(2)</td>
<td>Antineoplastic, antiadrenal</td>
<td>3</td>
</tr>
<tr>
<td><strong>Transporter proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Voltage-gated ion channels</td>
<td>29</td>
<td>Antihypertensive, anti-arrhythmia</td>
<td>181</td>
</tr>
<tr>
<td>Other ion channels</td>
<td>6</td>
<td>Anaesthetic, anti-arrhythmia</td>
<td>83</td>
</tr>
<tr>
<td>Solute carriers</td>
<td>12</td>
<td>Antihypertensive, diuretic</td>
<td>4</td>
</tr>
<tr>
<td>Active transporters</td>
<td>7</td>
<td>Antihypertensive, diuretic</td>
<td>63</td>
</tr>
<tr>
<td>Other transporters</td>
<td>3</td>
<td>Antihypertensive, anti-ulcer</td>
<td>19</td>
</tr>
<tr>
<td>Auxiliary transport units</td>
<td>10</td>
<td>Hypnotic and sedative, anti-anxiety</td>
<td>13</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme-interacting proteins</td>
<td>13</td>
<td>Anti-inflammatory, antineoplastic</td>
<td>84</td>
</tr>
<tr>
<td>Structural and adhesion proteins</td>
<td>11</td>
<td>Anti-inflammatory, glucocorticoid</td>
<td>36</td>
</tr>
<tr>
<td>Ligands</td>
<td>12</td>
<td>Antineoplastic</td>
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<tr>
<td>Other</td>
<td>15</td>
<td>Antirheumatic</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-inflammatory, antineoplastic</td>
<td>24</td>
</tr>
</tbody>
</table>
The druggable Genome – How many targets are there?

1st estimate:  

1. Identified protein families with evidence to be drugable: bind molecules with affinity $> 10 \mu M$.
2. Extrapolate to total number of family members in genome.  
   -> druggable genome
3. Estimate number of disease modifying genes  
   e.g. from gene knockouts

---

Table 1 | Comparison of the druggable genomes of selected eukaryotes

<table>
<thead>
<tr>
<th></th>
<th>Homo sapiens</th>
<th>Drosophila melanogaster</th>
<th>Caenorhabditis elegans</th>
<th>Saccharomyces cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of predicted genes*</td>
<td>~30,000</td>
<td>13,601</td>
<td>18,424</td>
<td>6,241</td>
</tr>
<tr>
<td>Number of proteins in proteome*</td>
<td>21,688</td>
<td>13,849</td>
<td>17,946</td>
<td>6,127</td>
</tr>
<tr>
<td>Number of estimated druggable targets</td>
<td>3,051</td>
<td>1,714</td>
<td>2,267</td>
<td>508</td>
</tr>
<tr>
<td>Percentage that are predicted druggable targets</td>
<td>~10–14%</td>
<td>12%</td>
<td>12%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Three hundred and seventy-six targets identified to bind rule-of-five-compliant drugs have had InterPro domains assigned. The prevalence of these InterPro domains in various genomes has then been determined. Twenty-three more bacterial and viral drug targets for which InterPro assignments could not be made have not been included in any of our analyses. *Data taken from InterPro, 29 October 2001.
Case study: Our own drug discovery program: Hu protein R

**Background:** We have identified a very interesting protein. It seems to have some disease relation and we are thinking of choosing this protein as our target for our drug discovery program. The protein is called HuR, a family of Hu proteins. It contains 3 domains, all from the RRM fold, a fold which binds RNAs. HuR binds RNAs (in a region containing a defined binding motif) and we can measure this interaction in-vitro using a fluorescence assay.
Case study: Our own durg discovery program: 
Hu protein R – Step 1 Target validation

Facts:

- suspected to play a major role in cancer development
- ubiquitously expressed
- very old protein family
- ARE pathway controls expression of 90% of all cytokines and chemokines

Q: is that already a good target?
What is needed for target validation?

A: No, many more steps do have to be established for target validation.
HuR– Step 1 Target validation checklist – initial

- Disease link - plausibility
- Genetics data
- Proteomics data
- Biomarkers
- Disease models
- in-vitro assays on the function
- Structural biology information
- Protein class

- RNA binding can be monitored in vitro
HuR in cancer

- Hu proteins originally identified in 1965 as tumor antigens in patients with cerebrellar carcinomas, carcinomatous neuropathies and systemic lupus erythematosus.
- HuR overexpression and increased activity of relevance in colon cancer\(^1\), ovarian cancer\(^2\), CNS tumours\(^3\), neoplastic lung cancer\(^4\), renal carcinoma\(^5\), prostate cancer, adenocarcinoma, papillary carcinoma, malignant melanoma, breast cancer, squamous cell carcinoma, …
- Increased HuR activity involved in the **onset**, **progression**, **metastasis** and **vascularization** of tumors.
- Expression level and cytoplasmic localization of HuR correlated with tumor grade, malignancy and survival.
- Proof-of-concept with siRNA knockdown of HuR in **in vivo** colon cancer model.

\(^1\) DeSilanes et al, Oncogene. 2003, 22(46):7146-7154
• ARE pathway controls expression of 90% of all cytokines and chemokines

• ARE mediated rapid decay compromised during allergic inflammatory responses (e.g., allergic asthma), leading to persistently high levels of cytokines and chemokines

• Knockout mice of HuR antagonist tristetraprolin (TTP) develop chronic inflammatory syndromes (cachexia, rheumatoid arthritis, autoimmunity, dermatitis, conjunctivitis) due to excess in TNFα, GM-CSF and Cox-2 (‘TTP-deficiency syndrome’)

• Phenotype can be prevented by weekly i.p. administration of TNFα antibody
HuR – Step 1 Target validation final checklist

- Disease link - plausibility ✓
- Genetics data ✓
- Proteomics data ✓
- Biomarkers ✓
- Disease models ✓
- in-vitro assays on the function ✓
- Structural biology information -
- Protein class -
Idialized Drug Discovery & development flow chart

1. Disease area basic research; Medical disease understanding
2. Assay strategy
   - Assay development
   - Assay validation
3. Screening
4. Hit confirmation, Hit validation «counter screening»
5. Compound repository Archive
6. Med. Chem. optimization
7. Hit Selection based on «lead criteria»
8. Hit Selection Hit to «Lead» early chemistry
10. Process development, Disease marker, endpoint definition
11. IND Investigational New Drug application
12. Clinical Phase I
13. Clinical Phase II
14. Clinical Phase III
15. NDA NEW DRUG APPLICATION
16. Clinical Phase III

Assay development and Screening

- "Screening" in this context is any form of testing molecules for a biological effect
- "Assay" is the testing method which allows to see the potential effect of a compound
- The Screening strategy defines what specific assay development is needed

How to probe Biology vs chemistry

- ~ 30 000 human genes
- 400 exploited for all current drugs, all belonging to 6 target classes

Chemistry Universe: ~ $10^{60}$ cpds
Different Screening approaches – non-target based

1. Phenotypic screening:
   Oldest method. Effect of compounds on cells/organism tested
   + holistic view of the system;
   + many proteins tested for each compound
   + in-vivo/cellular efficacy tested as well
   - Unfocused discovery of known compounds/targets
   - identification of target often extremely difficult
   - what if no clear target?

2. In-vivo model screening:
   Requires model organism with specific properties: e.g. Zebrafish development model
   + holistic view of the system;
   + ensure compound hits desired pathway
   + in-vivo/cellular efficacy tested as well
   - Complicated to set-up
   - Slow testing
   - target difficult to find
Different Screening approaches – target based

3. Cellular screening:
   a) generic reporter gene assay: a luciferase/GFP/etc. is cloned under control of a promotor which is linked to the activity of the protein of interest.
   b) specific cellular assay: e.g. T-cell activation, fluorescence based imaging, etc..

   + medium fast /rel. easy setup
   + cellular efficacy tested
   + automation

   - Off-target effects (other than desired target/multiple)
   - Limited in-vivo relevance
   - false negatives

3. In-vitro screening:
   a) enzymatic or competition screening
      + easily automated
      + fast
      + target focussed

      - Target needs to be an enzyme /or have natural ligand
      - Reductionistic (no in-vivo relevance per se)

   b) affinity based screening
      + very fast/automated
      + target focussed
      + generic

      - Hits selected for binding not biol. Activity
      - Highest degree of reductionism
Screening – miniaturization and automation of testing

- From early 1990s, Screening increasingly developed into an automated process with standardized elements and a focus on throughput
- Fluorescence developed into one of the key detection technologies

### The History of Microplate Formats

<table>
<thead>
<tr>
<th>Format</th>
<th>Wells</th>
<th>Compounds / day</th>
<th>Volume / well</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 wells</td>
<td>10.000</td>
<td>100-200 µl</td>
<td></td>
</tr>
<tr>
<td>384 wells</td>
<td>40.000</td>
<td>30-100 µl</td>
<td></td>
</tr>
<tr>
<td>1536 wells</td>
<td>200.000</td>
<td>2.5-10 µl</td>
<td></td>
</tr>
</tbody>
</table>
Liquid Handling (3) – Automated Systems

Key Features:
- Accuracy
- Precision
- Speed

Biomek FX (μL)

MITONA System (24 nL)

Cybi-Well (μL)

NPRS System (50nL)
Automated Screening Systems

Key Features:
Automation
Flexibility
Speed

FLIPR

Mark II SCARINA

ViewLux/LeadSeeker

Mark III SCARINA
**Key Features:**
Integration
Flexibility
“Big data” handling

**Project Proposal:**
web-based tool for all of Novartis research community
hyper-linked to chemistry/biology/literature databases
project management functionalities

**Project Tracking:**
Tool Prod. 1
Tool Prod. 2
Assay Dev.
HTS

ex pec. time
actual time

Slide: NIBR – DT LDC
Cellular assays

- A variety of different methods to generate a readout has been developed
- Most common:
  - Imaging assays after fluorescence staining («high content screening»)
  - FACS assays (for receptor binding)
  - Reporter gene assays – simple quantification of a fl. Reporter gene
  - Assays for GPCRs and ion channels based on functional fluorophores (see next slides)
  - many more...
Cellular screening – “high content screening”

General principle of HCS:
- advanced optics to scan wells very fast
- 2-3 color channels
- Automated pattern recognition and quantification software to compare intensities, etc's in different channels.
The over-expression of a transmembrane transporter, the ATP-dependent P-glycoprotein (Pgp), is one of the reasons for multi drug resistance (MDR). The Pgp pump actively decreases the intracellular concentration of the therapeutic agent.

Assay: Non-fluorescent calcein acetoxy methyl ester (CaAM) diffuses through a MDR cell containing the Pgp pump. Upon active transport, the ligand is extruded from the cell. Adding a Pgp inhibitor (e.g., verapamil, cyclosporin A) results in an accumulation of CaAM in the cytoplasm where it is hydrolyzed by intracellular esterases yielding fluorescent calcein. HCS readout parameter: total cellular fluorescence.
Cell viability assay

- In drug development, compounds may fail because of their toxicity => cytotoxic potential of a compound has to be assessed.
- Homogeneous cell viability assay: automated, simultaneous determination of the total number of live and dead cells with high throughput. Readout signal: total cellular fluorescence in green and red detection channel.
- Cells are pre-stained with 5-chloromethylfluorescein diacetate (CMFDA). The wells of 1536 NanoCarrier plates are preloaded with Ethidium homodimer. Ethidium homodimer diffuses through the cell membrane into dead cells, giving rise to red cell fluorescence (red = dead cells).
The β<sub>2</sub>-adrenergic receptor is an example for a G-protein coupled receptor which represents an important target for drug discovery.

Binding activity of compounds to this receptor is determined using a fluorescently labeled ligand which specifically binds to β<sub>2</sub>-adrenergic receptors on the plasma membrane.

In a second color channel the identity, number and location of cells is determined using a cell staining dye. HCS readout: spectral cell fluorescence intensity.

The ability of non-fluorescent compounds to compete for binding to the β<sub>2</sub>-adrenergic receptor with a specific ligand can be assessed with high throughput.
FLIPR: Fluorescence Imaging Plate Reader

Imaging: 384 wells recorded simultaneously
Integrated fluidics:
  injection of compound during measurement
Kinetic data (1 second updates);
detection of fast responses
Rich data output; possibility for multiplexing
  from same assay well (selectivity, toxicity)
Sensitivity (laser, CCD, patented optics)
Automated platform: 2 FLIPRs integrated
FLIPR: main applications

Targets
- G protein coupled receptors (GPCRs)
- Ligand-gated ion channels

Assay Types
- Measurement of intracellular calcium (Fluo-4)
- Membrane potential assay (MP dye)
- Other specific assays (e.g. pore formation via P2X7/ YoPro-1)

Assay Formats
- Discovery of agonists, antagonists, positive modulators
- Multiplex assays (Agonist/Antagonist, Agonist/Modulator, Receptor1/Receptor2)
- Agonist fishing in orphan receptor research
FLIPR: Calcium assay principle

Calcium assay principle:

- **Calcium**
- **IP₃**
- **PLCβ**
- **Esterases**
- **Gq-GPCR**
- **GPCRs**
- **Laser excitation 488 nm**
- **Fluo**
- **Fluo AM**
- **PtdIns DAG**
- **ER**
- **Multiple Well Average**

**Fluorescence emission:**
- Filter 510 - 570 nm
- (Ca²⁺-Fluo4 peak at 516 nm)
FLIPR: Membrane potential assay

Membrane potential assay

Fluorescence emission:
Filter 540 - 590 nm

Cell

Cl⁻ Channel

Depolarisation

Na⁺/ K⁺/ Cation Channel

Hyperpolarisation

Resting Potential

MP dye

Laser excitation 488 nm

Fluorescence emission: Filter 540 - 590 nm

Multiple Well Overlay

Cl⁻ Channel/Depolarisation

0µM

0.3µM

0.1µM

1µM

3µM

10µM

30µM

100µM

Time (seconds)

Fluorescence Change (Counts)
Reported gene assay principle

- **Promotor**
- **Target interaction**
- **Compound**

 Reporter gene (e.g. encoding GFP or luciferase)

- **DNA**
- **mRNA**

A reporter protein
Amount is easily measured (e.g. GFP by fluorescence)
In-vitro assays

- Generally 2 types: competition screening & direct binding assays

  - **Competition screening**: based on the displacement of a ligand from the target protein. Ligand can be either a natural or a «surrogate» ligand.

    Measurement format:
    - most common: fl. based for screening
    - alternatives exist: e.g. chip-based biacore

  - **Direct binding assay**
    - Formats: a) fl. Ligands (uncommon)
      - b) ligand immobilized (bead based, chip based)
      - c) protein immobilized (chip, bead e.g. alpha screen)
    - d) size-exclusion
Practical approach for fluorescence competition assay

Target interaction:

hDm2 (proto-oncogen) interacts via a helix with p53 (tumor suppressor)

Goal: find inhibitors of hDM2-p53 interaction

Step 1: generate «surrogate ligand»: natural or already known ligand here, take p53 helix and use fl. labeled form.

Determine best labeling site
Step 2: measure affinity/binding of ligand to protein

Step 3: perform assay in presence of high concentrations of compounds (to be screened)
Assay set-up:

![Graph showing receptor concentration vs percent binding with different conditions: Absence of cpd, Presence of cpd 1, Presence of cpd 2, Presence of cpd 3.]

Normally, concentration of Protein is chosen to be at 50-80% saturation with signal. If compound is active, it will reduce the signal depending on its affinity.
Affinity based screening using size exclusion

1. Incubation
Pool of 400 compounds + protein

2. 96-well format SEC
Separation of protein/ligand complex from non-binders

3. LC/MS analysis
Mass spectrometry of ligand (binder)

4. Database Query
Identification of binder

“Sandwich” Technology

Loading
Protein + compound pool

SEC

Collecting
Protein + Ligand

Source: I. Muckenschnabel et al. (2004)
**Example:** Using simple dialysis for analyzing direct binding

**The process:**
- Quality check @HPLC first
- Dialysis 24h
- Sample 50µl + 10µl ACN in vials
- HPLC \rightarrow upper and lower conc.
- Calculate partitioning \( p_b, p_c, p_s \)

**The principle: Diffusion vs binding**
- Exchange between 3 “compartments”: V1, V2, and target protein
- Described as two parallel pseudo-first order binding reactions; Diff.eq. solved
- Concentration ratio between upper and lower compartment \( \rightarrow \) partitioning \( p \)

\[ p = \frac{c_{1f} + c_{1b}}{c_2} \]

\[ t_p = \frac{1}{k_{12}} \]

\[ c_0 \]

\[ c_{upper \ well} \]

\[ c_{lower \ well} \]

\[ \pm \ target \ protein \]

\[ + \ target \]

\[ control \]

\[ cmpd \]

\[ binding \]

\[ p_c > 1 \quad p_t > p_c \quad p_t > 1 \]

\[ 20 \quad 15 \quad 10 \quad 5 \]

\[ time \ [h] \]
**Example:** Dialysis results with 4 different compounds:

- **Cpd 1:**
  - Non-dialysable
  - $p_c >> 1$
  - Aggregated
  - $p_c = 52$

- **Cpd 2:**
  - Dialysable
  - $p_t = p_c$
  - No binding
  - $p_t = 1.13$

- **Cpd 3:**
  - Dialysable
  - $p_s = p_t > p_c$
  - Specific
  - $p_t = 2.97$
  - $p_s = 16.1$

- **Cpd 4:**
  - Dialysable
  - $p_t > p_c$
  - Binding
  - $p_t = 2.49$
  - $p_s = 1.57$

HPLC trace integration $\rightarrow$ partitioning

- Upper peak area
  - $p_c = \text{control}$
  - $p_t = \text{target}$
  - $p_s = \text{specificity}$

- Lower peak area
  - $p$
Assay quality parameters

- High throughput screening generates large amounts of data sets
- The default result is a negative finding (no activity, baseline)
- Hits of different activity levels will deviate from standard negative results
- HOWEVER, data set is gauss distributed.

- Problem: Reliable detection of hits.
- Depending on assay:
  1 – 0.01% hit rates are std.
  -> 100 000 compounds/day
  -> 10 – 1000 hits
  -> threshold setting is difficult!
How to judge if an assay is suitable?

a) generate standards using a positive and a negative control

- Positive control
- Negative control

False positives: a lot of additional work
False negatives: a missed opportunity
Assay quality parameters

- Zhang et al. suggested a simple criterion: $Z$-factor

$$Z' = 1 - \frac{3(\sigma_{\text{high}} + \sigma_{\text{low}})}{\mu_{\text{high}} - \mu_{\text{low}}}$$

$\sigma = \text{Standard deviation}, \ \mu = \text{mean}$

- For a good assay, $Z' > 0.5$

Basis for $Z$\textquotesingle factor = Gauss distribution

Histogram of negative controls

At an inhibition of $> \mu + 3\sigma$ the probability to find a positive hit is $> 99\%$

At the inhibition level of $> \mu + 3\sigma$ the probability for a false positive is $< 1\%$
Rational for Z`factor

For a good assay, the assay window must be at least 2x of $(3 \sigma_{posC} + 3 \sigma_{negC})$!

$Z` = 0.5$ means Assay window is exactly 2x of sum of 3x sigma $posC + 3$ sigma $negC$
Assay quality – multiple dimensions

- Assay quality can be improved by measuring more than 1 readout!
Real life example of screening data

**Assay development data:**

**Pos. & neg. control**

**Time-Resolved Anisotropy (cTRA)**

Neg. control

Pos. control

HTS Hits
Fluorescence as essential detection technology in Drug Discovery
Fluorescent dyes

- A fluorescent dye = a «molecular maschine» which absorbs light and emits light at a higher wavelength than it takes it up (stokes shift).
- Phenomenon is coupled to molecular structure.
- Pi-electrons and «non-bonded orbitals» absorb light, molecules get «colored», but only if radiative decay occurs, fluorescence emission is seen.

![Chemical structures of fluorescent dyes with wavelength ranges](image)
Fluorescence Spectroscopy

Fluorescence

Absorption

Intersystem Crossing

Vibrational Relaxation

Phosphorescence

Energy Transfer

Collisional Quenching

Chemical Reaction

Intersystem Xing

Internal Conversion

HEAT!
Steady State and Time-Resolved Fluorescence Spectroscopy

Steady State Fluorescence Spectroscopy

Time-resolved Fluorescence Spectroscopy

\[ I(t) = I_0 \sum a_i \exp\left(-\frac{t}{\tau_i}\right) \]
Advantages of fluorescence

• Homogeneous Mixtures of Reagents
• High Sensitivity
• True Equilibrium
• Wide Range of Solution Conditions
• Picosecond to Microsecond Timescale = Timescale of Dynamic Events in Molecules:
  – Rotational Diffusion
  – Solvent Reorientation
  – Energy Transfer
  – Motion of Domains
Push-pull $\pi$-electron systems are particularly efficient for fluorescence.
Example: Cy5 dye

- Solubility
- π-electron system
- Attachment point
Fluorescence wavelength change with pi-electron system

Cy5
Ex/Em = 649nm/670
Quantum yield water = 0.28

Cy3
Ex/Em = 550nm/570
Quantum yield water = 0.04
Important criteria and properties

- Type of assays (Cellular, in-vitro, Intensity, Anisotropy, Lifetime)
- Excitation/Emission wavelength
- Solvatochromism
- Solubility
- Size
- Cell-penetration, subcellular localization
- Chemical Stability
- Type of functionalization (see chapter Bioconjugation)
- Price
Most important class of fluorescent dyes and their properties

**Oxazines**
- Small, pos. charge

**Cyanines**
- Chemically sensitive

**Rhodamines**
- Chemically stable, hydrophobic

**Fluorescein**
- Photosensitive

**Alexa-488**
- Photostable, 2x negatively charged
Quenchers

- Have similar structural features as fluorescent dyes (extended $\pi$-system) but also contain a group which allows them to pass the energy on to the environment (solvent) without emitting photons.

Example:

BHQ-1:

Nitro group is «quenching group»
Fluorescent Labels on Biomolecules

**An Important Tool in Pharmaceutical Research**

The Many Benefits of Fluorescent Labels:

- easy to handle in standard research laboratories
  
  (via PMT- or CCD-based detection equipment)

- relatively cheap reagent costs
  
  (compared to radionucleotides; cf. waste disposal/containment)

- large linear dynamic range for detection
  
  (well suited for detection)

- high sensitivity for detection
  
  (dependent on readout technology)

- well suited for miniaturization/parallelization
  
  (increasing importance for Pharmaceutical & Basic Research;
   many examples from Systems Biology, Genomics, Proteomics, HTS)
High-Throughput-Screening (HTS) example:

Biochemical Assays

Protease
(e.g. Alzheimer’s, HIV, HCV, Cancer)

fluorogenic protease substrate

Boc-Ile-Glu-Gly-Arg-AMC

fluorescence

λ_{ex} = 342 nm
λ_{em} = 440 nm

readout signal: fluorescence

0 2000 4000 6000 8000 10000 12000 14000 16000 18000 20000
0 30 60 90 120 150 180
Time [min]
RFU

fluorescence

fluorescence

fluorescence

fluorescence

fluorescence

fluorescence
Basic principles of fluorescence based protease assays

(a) Fluorescence intensity assay in which a fluorogenic group is linked to the carboxyl end of a peptide via an amide bond, and its fluorescence increases upon release by the action of a protease; (b) Resonance-energy-transfer-based assay in which a FRET signal can be detected when the donor and acceptor are in close proximity. The pair separates upon peptide cleavage and the FRET signal decreases; (c) Dual-label quenched-pair fluorescent assay in which the fluorescence intensity of the reporter is suppressed by the quencher because of its close proximity. The pair separates upon peptide cleavage and the fluorescence intensity from the reporter group is significantly increased; (d) Fluorescence polarization assay in which the substrate and the product give different emission polarization signals because of their different sizes ("digestive" fluorescence polarization assay).

Detection of Fluorescence Intensity

The fluorescence contribution comes from the entire well

Interference from:
- surface interactions
- fluorescent compounds
- light-scattering particles
- inner-filter effect

< ~ 5 µl
# Main Assay Interferences in Fluoro-/Luminescence

<table>
<thead>
<tr>
<th>Assay Interference</th>
<th>Main Cause</th>
<th>Fluorescence Effect</th>
<th>Luminescence Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner Filter Effect</td>
<td>Anything in the assay that absorbs excitation light (e.g. buffer components, colored compounds, biological tissues, plates, etc.) and reduces the amount of light that reaches a tracer, including absorption by the tracer itself (e.g. at high concentrations) or reduction in the light emitted by the tracer</td>
<td>↓ Fluorescence Intensity</td>
<td>↓ Emitted Light</td>
</tr>
<tr>
<td>Quenching</td>
<td>Non-radiative deactivation of the excited state of the tracer</td>
<td>↓ Fluorescence Intensity</td>
<td>↓ Emitted Light</td>
</tr>
<tr>
<td>Auto-Fluorescence</td>
<td>Anything in the assay, other than tracer, that adds fluorescence intensity at the assay wavelengths (e.g. buffer components, compounds, biological tissues, plates, etc.)</td>
<td>↑ Fluorescence Intensity</td>
<td>← None</td>
</tr>
<tr>
<td>Light Scattering</td>
<td>Turbidity resulting from particulates (insoluble compounds, but also some beads and cells, etc.) in the medium</td>
<td>↓ Fluorescence Intensity</td>
<td>↓ Emitted Light</td>
</tr>
<tr>
<td>Photo Bleaching</td>
<td>Light-induced reaction with tracer (e.g. oxidation) resulting in loss of ability to fluoresce or absorb light</td>
<td>↓ Fluorescence Intensity</td>
<td>← None</td>
</tr>
</tbody>
</table>