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PHARMACOTHERAPY OF BIOLOGICAL DRUGS

IMMUNOGENICITY (5° lezione)

Important to know wich kind of consequences it may trigger.
When you think about reasearch of innovative drugs, there is always a focus on the mechanism of action ad the translational process to get the drug to the site it needs to interact with.

In principle the toxicity is lower because they are more specific on target.
Inversely if therapeutic proteins trigger an immune response, then it would be a problem.
Recombinant proteins may be used as diagnostic tools and even here immunogenicity may present some issues.

THE KEY TO THE PRODUCTION OF THESE PROTEINS IS THE ABILITY TO DEVELOP PROTEINS THAT ARE IDENTICAL TO THEIR COUNTERPART.
Sometimes there are exogenous proteins that are therapeutic agents, like enzymes, that are required to have an effect, like anti-coagulants.
You expect them to have a strong immunogenic effect.
Endogenous proteins, or recombinant copies of exougenous proteins, may also have immunogenic effects.
This may deeply impact the therapy.
It needs to be controlled during the development of the drug.

ADA (anti-drugs antibodies)

This is the issue**: TREATED PATIENTS WILL START PRODUCING ANTIBODIES AGAINST THE GIVEN DRUG**, and those can bind to the drug (**Babs**) or even neutralize it (**Nabs**).

- **Babs (binding antibodies)**
  Are ab (antibodies) specifically against our drug, theu bind to our drug, but without directly impacting on the farmacodynamics, **but for sure altering the pharmacokinetic aspect**, this can lead to an alteration of pharmacodynamics.
  The binding doesn't prevent the effect of the durgs, they bind to a specific position but they doesn't alter the ligand receptor interaction of the drug.

- **Nabs (neutralizing antibodies) =** they alter the ligand-receptor interaction od the drug
  They prevent the protein from eliciting the action.
  For example if you are giving eritropoietin to a patient to have replacement therapy and he produces Nabs, **they will bind to erytropoietin and inhibit it's action**.
  Fortunately, if we are talking about endogenous or recombinante versions of endogenous proteins it is less likely that Nabs are produced (more likely that ADA are Babs rather than Nabs).

WHEN THE PROTEIN IS EXOGENOUS, THEN EXPECT NABS.

It's important to consider that **NABS MAY CROSS-REACT WITH ENDOGENOUS COUNTERPARTS**.

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- **AS AN EXAMPLE, WE WILL FOCUS ON ERYTHROPOIETIN**
  People affected by an eritropoietin-related pathology suffer because **the erythropoietin in the body is not enough**, so the common therapy is to give it to them.
  If Nabs develop against erythropoietin, they will not distinguish between native erythropoietin and the drug, **so the action of the erythropoietin will not work and the conditions will worsen.**
  This is the danger to give patients the recombinant form of the endogenous protein, that the **IMMUNOGENICITY MAY AFFECT SAFETY AND EFFICACY.**

MOLECULAR MECHANISMS

The classic immune response is easy.
IT'S THE CLASSIC RESPONSE TO KNOWN SELF ANTIGEN.
If you have therapeutic proteins that come from animal, plant or microbial origin, **they are likely to trigger the immune response**, **as any non-cell immune antigen**.

The first injection of the proteins is enough to trigger this type o responds.
This response leads to **FAST AB FORMATION**, the duration of these **ab** is long, **mainly Nabs are produced, becouse the cause of production is these antigens.**
The result is a loss of efficacy.

One important thing is that some substances important in the human body are produced in E.coli. Because it is a very convenient way to produce a protein (if post-translation modification aren't needed), but:
what if E.coli components or products are not purified completely in the production of this drug?
You risk to have immunogenic components in your drug.
Bacterial leftovers may have effect on the immune response and activate the immune system against your drug.

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BREAKING OF IMMUNE TOLERANCE

ADA developed against proteins that are identical or homologous to their endogenous counterpart.
Their production is, maybe, **due to the breaking of IMMUNE TOLERANCE:** the immune system loses tolerance to a self-component.
It's a slow reaction usually occurs over the prolonged course of replacement therapies.
The longer these therapies last, the more likely this phenomenon occurs.
Usually when Babs are produced, stopping the treatment, stops the production of ADA.

Mainly this is caused by impurities, sometimes it is unknown, but quite often it has to deal with:

- **THE FORMULATION**
- **THE PREPARATION**
- **THE STORAGE**

For example, in the industrial process some excipients may be changed to optimize the production (necessary for upscaling) **and this may affect this outcome**.
The classic immune response is triggered by APC (antigen presenting cells) that use MHC-II to present the antigen to the dendritic cells and trigger the acquired immune response T cell and B cell dependent **(These are typically Nabs)**
Contaminants may trigger this response.

IF YOU HAVE A CONGENITAL DEFICIT OF A FACTOR, not like the reduction of erythropoietin production, so the absence of this compound.
That individual did not develop self-tolerance towards that factor, because it was never there.
Because it was never produced during the development of the immune system it was never selected as a self-antigen, so, **if we administer the missing protein as a drug, it is more likely that Nabs will be produced.**

AGGREGATION can affect protein folding of the drug, this mis-folding can present part of the protein that can elicit the immune response, or drug aggregation can form specific pattern that can activate immune response.

THE PRODUCTION OF ADA CAN BE T CELL DEPENDENT AND T CELL INDEPENDENT.
If the terapeutic protein is purely biological they may be ingulfed by antigen-presenting cells and this leads to the production of ADA, typically IgG.
In the T cell-independent pathway B-cells are activated directly by the protein, it is tipically caused by aggregation (really impactful on immunogenicity).

B cells are specialized in recognizing cross linking antigens, **because many pathogens have the tendency to have these multimeric antigens on the surface**.

So trough the clustering of B cell receptors and the recognition of a multimeric antigen (the aggregation of biologic drugs may mimic this multimeric antigen) the immune response can be activated.

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The key mechanism allowing the breaking of immune tolerance is multimeric antigen presentation.
In a very confined space there is a high concentration of antigens.

This is what may happen when some specific pathogens are recognized by B cells, but also when you have aggregation or when the protein is unfolding or partial unfolding of the therapeutic protein may bring aggregation (while unfolding the protein exposes the hydrophobic domain, that attracts other hydrophilic domains leading to aggregation) **wich results in proliferation and activation of B cells that produce ADA.**

The immunogenicity we are talking about is unwanted.
There cases where drugs are used to strengthen the immune response (like administering cytokines).

Figure: ***FACTORS AFFECTING UNWANTED IMMUNOGENICITY***
And what if, during the storage period, **these components and the material interact to induce the release of some leachates from the rubber stopper or cap in solution**.

This will be injected in the patient and may help in the unwanted immunogenicity.
Also, containers may impact on the folding and the aggregation.
It is important to consider the packaging when upscaling.

If you have in your plotting sequence a higher proportion of non-self sequences this is immunogenic.
The first antibody drugs were murine, they were therapeutic, but immunogenic.
In the structure of the Ig the murine part was reduced more and more, until now that it does not contain murine parts anymore.

GLYCOSILATION PATTERN MAY AFFECT IMMUNOGENICITY
Your recombinant protein needs to be properly glycosylated.
Folding is also important because misfolding may expose non physiological epitopes that may trigger immunogenic response.
This because our immune system is trained to recognize out proteins as they are folded in a certain way.
IF THEY ARE FOLDED DIFFERENTLY, IT WILL BE ALLARMING.
The patient background is also important (genetic, other treatments, the type of HLA molecules…).

SAME DRUG + DIFFERENT PATIENTS = DIFFERENT IMMUNOGENICITY

PRODUCT RELATED FEATURES

- **STRUCTURE**
- **FORMULATION**
- **PURIFICATION**

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Alterations in the primary sequence are not enough to predict if they will influence immunogenicity.
In recombinant insulin 1 aa change can make the product immunogenic, but it isn't the rule.

- **IFN-alfa,** there is a consensus sequence that pairs the aa most widely represented if you consider the variants present in human.
  For each aa position you have the most represented one.
  It does not exist in nature, it's a consensus, meaning that if you compare this general consensus to the variants there are 10 to 23 alterations, **but this consensus IFN-alfa is not immunogenic**.

- **GLICOSILATION** affects molecular stability, *in vivo* activity, serum half-life.
  Usually glicosilation is protective against immunogenicity**, because it may shield certain epitopes that may cause an immune reaction** and **they may increase solubility, preventing aggregation**. **ALTOUGH A GLICOSILATION PATTER DIFFERING FROM THE BODY'S STANDARD MAY BE IMMUNOGENIC.**
  INSULIN IS NOT GLICOSILATED.

- **PEGylation** is the most successful approach to increase the plasma half-life because it decreases the rate of clearance, shields proteins from proteolytic enzymes, reduced immunogenicity.
  BUT sometimes PEGylation increases immunogenicity.
  - **Investigation on drugs: PEG-rhMGDF**
    rh stands for recombinant human and MGDF stands for Meta-cariocytes growth and development factors.
    The PEGylated form is immunogenic, is thought thai is caused by longer half-life of the drug.

When we talk about therapeutic proteins the administration is always parenteral.

- Endovenous administration was observed as less immunogenic
- Intramuscular administration
- Subcutaneous = is very easy and can be done at home directly by the patient.

DETECTION

No standard assays have been developed.
There is no standard, everyone does it as they want.
This might be a problem in comparing studies.

The ideal procedure should include different assays.
Each assay has pros and cons, so that the combination of more assays can lead to bigger advantages.
Binding assays can identify and quantify the presence of ab
But that's it. it won't tell you if they are Nabs or Babs.

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YOU NEED TO DO A SUBSEQUENT NEUTRALIZATION ASSAY.

In **ELISA** the drug is a monoclonal ab coated on your well.
You have another version of ab labelled with fluorescent or enzime to give a measurable signal and you put in the well the patient's sample.

If there are ADA they will recognize the ab drug on the well **so it will be recognized by anti-human IgG Fc, the secondary ab.**

- **RIFORMULATING**=> you have your drug: an ab against a particular cell tumor.
  You coat it on the plate.
  If there is ADA in the patient's sample, it will bind to the plated ab.
  In the solution we also added a secondary ab, containing a fluorophore, that binds to the **Human Fc**, so it will bind only if ADAs are present.
  Then the plates will be washed and measured the luminescence.
  You will not distinguish Nabs from Babs.

Another ELISA with same drug in the same plate.
IN THE SAMPLE YOU MAY HAVE ADA.
The detection is different.
You don't use a secondary anti-human ab, **but by a fluorescent version of our drug**.
This will be recognized by the second arm of IgG.
This procedure is liable to false negatives, because if the **ab binds somewhere**, **is not able to breach your labelled drug, so will not be recognized and you won't have luminescence.**
=>false negative.

RADIOIMMUNO ASSAY:
In solution you mix your radioactive drug with what you extracted in the lab.
If there are ADA they will bind to the radioactive drug in the solution.
Then you add **ab against the light chain of human**.
They will form a bigger immunocomplex.
Then you centrifugate, separate the immunocomplexes and measure the radioactivity.

Surface plasmon resonance (SPR)

Allows not only to detect the binding, but to measure affinity. Because you can measure association and dissociation to the drug.
If the antibody binds to the antigen (our drug) the resonance will change in a measurable way.
Resonance depends on the size of the molecules attached to the surface.

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It could be late. What about some predictive methods to anticipate the insurgence of immunogenicity? The likelyhood of insurgence? We already discussed the presence of epitopes that can activate the immune system, these epitopes are described in databanks and can be used to predict the insurgence of immunogenicity by an algorithm in silico. These results do not produce certainty, but the probability of it happening.

IN VITRO EXPERIMENTAL ASSAYS:

You can see if your component can activate in vitro dendritic cells.
The problem with these approaches is the fact that they are time-, resources- and effort-consuming. Can't be scaled up.
Also the results sometimes are not clear enough and you need to combine various assays to get your answers.
Usually in SILICO METHODS are used first and then further investigated using in vitro and in vivo methods.

- **You need to be careful of the animal model** because they can be used to predict the immunogenicity of microbial proteins or protein that display a high interspecies homology, but they cannot be used for human proteins, they will see them as non-self!
- **IN SILICO PRREDICING ALGORITHMS =** mathematical models predicting mAb formation

IMMUNE TOLERANT ANIMAL

Exposing the mouse to high amount of protein for a prologue time, **they will become tolerant to the protein**.
The problem is that you need a huge amount of protein just to make the animal tolerant.
After the animal becomes tolerant you don't expect immunogenicity unless **the protein is immunogenic in and of itself.**

- **Transgenic mice** may be used, **they produce natively the protein, so it is considered self and does not activate the immune system if it isn't immunogenic**.

PRCA = ***pure red cell aplasia***, when you have Nabs cross reacting against the endogenous erythropoietin.

DRUG DISCOVERY PROCESS

Drug discovery is a process by which drugs are designed and discovered through therapeutic target identification.
First of all, you must have a target.
Then, you can make the assays, thousands and thousands of compounds against this target.
We don't have a drug against it, so we have to develop it.
We start with the HITS (**molecules that hit he target**); then, **we eliminate the majority of the HITS that are not effective**.
We pass from the HITS to the LEADS.

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- We have the **Exploratory discovery, the early discovery, and then we have the HITS**.
- **Then**, HIT ASSESSMENT, Evaluation of the HITS, Elimination of the Majority of the HITS, and then the LEADS.
- We move to Preclinical development, then Clinical PHASE I, II, and III ad we arrive at Phase IV.
- **At the bottom of this picture, you can observe that you started from millions of compounds, then you discovered HITS** (thousands of them)
  We move to the Hit assessment and then to the Leads compounds.
- Finally, we optimize the Leads.

THIS IS THE DRUG AND DISCOVERY PHARMACEUTICAL-PROCESSING BIOTECHNOLOGY INDUSTRY.

- **GMP** = good manufacturing practices
- **GLP** = good laboratory practices
- **GCP** = good clinical practices

From company the DRUGGABILITY is the starting point to the development of a marketable product.
Drug discovery process display high costs, low productivity, very low overall success rate.
Failure is attributed mainly to dismal success rate in phase 2 clinical trials.
Failure reasons are quite often related to lack of confidence in lead compound selection, lack of understanding the pathophysiology and insufficient safety, **SO HIT TO LEAD STRATEGIES BECOME CRUCIAL.**
THE HIT-TO-LEAD phase, identify lead compounds that will not fail in more expensive later stages od drugs development.
Integration if chemical biology, molecular screening, ADME studies **IS CRUCIAL**
Forward integration of preclinical studies into HIT-TO-LEAD optimization phase may be strategic to conquer the so called "valley of death".

THE SLIDES REPORT A STARTING MOLECULE WHICH IS THE TARGET AND THE HITS.
Then you have to apply a series of hots to the target and check which one can work.
The hits can be an antagonist or an agonist.

- **AGONIST**: mimic of the endogenous ligand
- **ANTAGONIST**: blocks the endogenous ligand

CRITERIA TO SELECT AN IDEAL LEAD SHOULD BE SELECTED A PRIORI, there are decided after the hits, so when we have our defined HITS and we want to select the LEADS (for example <500 kda)

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WHAT WE NEED TO STUDY?

- **POTENCY**: first parameter to study. **A hit is potent if can block the target also at little concentration** (low amount of hit can block the kinase of the example).
- **EFFICACY**: it refers to the quantity of the target that is blocked, so you can draw a graph with the increasing number of hit concentration on the x, and the percentage of inhibition on the y.
- **SELECTIVITY we want to block a specific compound.**
- **CYTOTOXICITY we have to test it not only in test tubes but also in cells**.
  We have to prove that the model doesn't kill the cells, otherwise it is useless.
- **DESIGN & SYNTHESIS** we have to produce the antagonist of the target, you have to set up the correct design and synthesis also taking in mind **physico-chemical proprieties** that can influence it.
- **METABOLISM**: we have to understand how long the molecule is effective. If the molecule is metabolized quickly and is destroyed is useless.
- **BIOAVAILABILITY:** how much of the drug is absorbed and reaches the target
- **Cyp P450 inhibition:** Cytochrome P450 is the most important metabolizer for drugs. **If the compound inhibits the cytochrome, we can prevent the metabolization**.
- **PERMEABILITY**: influence the absorption of the molecule.

HETEROGENEOUS VS HOMOGENEOUS ASSAYS

- **HOMOGENEOUS ASSAYS**, these assays are very fast, and you can make for hundreds and hundreds of compounds, Homogeneous assays are collected, mixed and measured, **everything is carried out with the same well, the same place**.
- **HETEROGENEOUS ASSAYS** involve multi-transfer, multi-processing steps (the more steps you do, the more easily you can make a mistake).

RADIOACTIVE ASSAYS

The main radioactive assay is the **SCINTILLATION PROXIMITY ASSAY**
You have the beads (spheres) covered by scintillant.
The scintillant is a compound that can multiplicate the radioactivity signal emitted by radioactivity particles.
Instead of checking for gamma particles, **this assay checks for beta particles**, but it is the same; alfa, gamma, and beta have the same origin as the I^{125}.
The sphere is covered with beads, and it is covered with scintillant.
YOU HAVE THE ANTIBODIES WITH SCINTILLANT; then, the insulin is no more radioactive and binds to the beads.
The insulin bind to the antibody and the antibody is bound to the bead.
If the radioactive insulin joins to the antibody, **you will have a signal.**
You have the signal because the Radioligand bound is in close proximity stimulating the bead to emit light. It is the same system as the insulin method, indirectly proportional.

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In the presence of MG-ATP, kinase can bind the phosphate.

To see this activity, you use the SPA activity; the antibody will bind to the phosphopeptide product.
These experiments depend on the availability of the antibodies; **without antibodies, you cannot do anything in the pharmaceutical field**.
If you go to the pharmaceutical/medical science, the antibodies (monoclonal antibodies) are always present.
Not all antibodies all the same; you can have antibodies that recognize different functions of the same protein.
Each antibody will be against a different region of the same protein (or different); so you can discover the best antibody for your study (the affinity is important for antibodies).

FLASH-PLATE

The target protein is immobilized in the wells of Flash plate coated with scintillant.
Here there is no beads.
We have the well covered with scintillant.
You have the radio ligand that is the star, binding with the proteins.

BETA-GALACTOSIDASE ASSAY

Beta-galactosidase is a glycoside hydrolase enzyme that catalyzes the hydrolysis of beta-galactosides into monosaccharides through the breaking of a glycosidic bond.
The beta-galactosidase assay is used frequently in genetics, molecular biology, and other life sciences.
IT CAN BE USED AS A BIOLUMINESCENT REPORTER: enzyme fragmentation and after complementation.
The enzyme can be divided into two form portions that are for different sequences, but to work as an enzyme, the form elements should be close together.
You have a substrate that is covered by a product, the product can be from the enzyme activity, but to get this result all the form parts of the enzyme must be put together. You can separate the enzyme as an acceptor sequence, separate from a donor system.

When you put together the donor with the acceptor, you reconstitute the full portion of the enzyme; and you get the product (with the identification). You need the donor enzyme and the donor ED-cAMP to get the signal.

The cAMP is measured as the target of this experiment.

- If you add the cAMP antibodies (B), the complex of the antibodies+cAMP won't bind the beta-galactosidase.
  So, the enzyme acceptor (EA) **remains not colored**, the enzyme doesn't join because the cAMP is not available to join to the enzyme.
- Another experiment (C), you can make a competition between ED-cAMP against the acceptor enzyme, so the antibodies bind to this complex.

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If the cAMP joins to the antibodies, it will let the production of the substrate.
Your sample is cAMP; if your reaction produce cAMP, it will produce the signal. You see the activated enzyme produces the ligand of four different sequences.

A common procedure in drug discovery is **THE FLUORESCENT ELISA**, in which fluorescent molecules are excited with polarized light.
So you need a machine capable to produce polarized light in order to excite fluorescent molecules.
Then excited molecules can emit radiation in the same plane as polarized light.
SCHEME OF COMPONENTS:

- **Excitation polarizer** à source of polarized light
- **Sample**
- **Emission polarizer** to register the emission of polarized light

If the fluorescent ligand is bound to a low molecular weight molecule it rotates rapidly and is oriented randomly so that the light is quickly depolarized.

In the following example, we see a polarized light that excites a fluorescent molecule, if it has a low molecular weight, **it rotates very fast**, and light loses polarization.
If you add to the molecule a high molecular weight antibody it reduces the rotation, and the light remains polarized.
Also in the following image, **we see a depolarized excitation light, which is polarized by a polarization filter.**
Then we have 2 different possibilities:

- **Slow rotation**: the enzyme allows a slower rotation, and the polarization is not lost.
- **Rapid rotation**: the light is depolarized if you separate the rapid rotation molecule from the enzyme. The fluorescence is not stable ad the emitted light is depolarized

So you need a fluorescence small molecule bound to a bigger molecule that is generally protein, like an antibody.

REPORTER GENE ASSAYS

Are based on the measure of:

- Luciferase
- B-lactamase,
- B-Galactosidase.

Luciferase is important because this enzyme allows fluorescence.
It has a response with different illuminance, allowing luminescent assays.

If you want to study second messengers that activate transcription of GPCR, you use a plasmid.
You have a plasmid; inside the plasmid, you put the sequence of the reporter gene (Luciferase) and use the promoter as a control.
In the case of the GPCR, the promoter can be the cAMP, t**he adenylate cyclase is the enzyme that produces cAMP, so we can measure the cAMP formation**.
To measure the levels of cAMP**, you have to assay the mRNA of the cAMP**.

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You can elevate the nuclear levels of cAMP mRNA, but you can do another thing simpler.
You can take the promoter region and put it inside the plasmid, so, you can activate the promoter of the cAMP and you measure the formation of Luciferase.
For this reason, Luciferase works for the reporter gene assay

HiBiT TECHNOLOGY

Relying on enzyme complemantaion t the enzyme that is going to be complemented is a nanoluciferase, we have a fragmente that is a dono anch ethe other is the receptor, the peak Is very small, the size is very important.
We can use the tecquineque in protein-protein interaction but you can thing about this tye of approach, thei are so small that ou can thinks about taggin molecules without altering their activity.

QUANTITATIVE SYSTEMS PHARMACOLOGY AND SCREENING OF INNOVATIVE MOLECULES
An approach that might be useful is quantitative system pharmacology, that combines computational and experimental methods to apply **new pharmacological concepts to the development and use of innovative drugs**.
QUANTITATIVE SYSTEMS PHARMACOLOGY is a pretty recent concepts that puts together system biology and quantitative pharmacology,

- the first one aimed to characterise biological networks in order to build models that can describe complex system, integrating the missing information that at the moment we have in the field due to inappropriate model and acting in a qualitative way,
- while quantitative pharmacology seeks computer aided modelling and simulation to increase the understanding of pharmacokinetics and pharmacodynamics, the two pillars that influence the effect of every drug, to aid in the design of pre-clinical and clinical experiments.

THE IDEA IS TO DEFINE THE MODEL SCOPE, so the therapeutic field and objectives, by incorporating the physiological pathways **the biological and pharmacological processes**, etc, to then build this model collecting all the information and developing mathematical descriptions and algorithms which describe the system well enough to help us predict the interplay between drug and the pathophysiology of the disease; **in this way we have a more complex and comprehensive process**, that is less linear than the traditional pipeline and combines various phases together, to improve the success rate.

QSP approaches wants to fully and quantitatively understand and describe the multifaceted connections between target activation and the resulting effect in a complex system

This approach has its challenges and limitations:

- **OMICS DATA,** while very useful and important, is not often included in the model, since it is very complex and difficult to implement in a system that is already quite complex by itself. The same can be said about some mechanistic understanding, that is not available yet and so can't help in adequately describe the complexity of the system of interest, and individual-level clinical data, that for a lot of recent fields is not present yet.

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- **INTEGRATION** is an issue also for various data from pre-clinical models, clinical settings and in general experimental data, since QSP is highly based on quantitative results that are sometimes missing and the framework of all biological interactions is not fully integrated yet.

ANALGESICS OR PAIN MEDICAMENTS are in fact a good example of the complexity problem in some systems:
Chronic pain affects 1 in every 5 individuals in Europe, and 60% of the patients report an inadequate efficacy of the analgesics medicaments, due to either inefficiency or adverse effects.
We are clearly missing some pieces of information in the comprehension of this system: each tissue is in fact composed of different cells, and the same drug may act differently in each of them, resulting in an alternate modulation and different final outcome.

In addition to this we also need to consider **that the cells are not single systems, but part of a more complex one, interconnecting and spreading the repercussions between one another**, all of this in an actual human organism of a patient that could be different from another patient.
While personalised therapy for each one of us is still an utopy, QSP could help us at least identify subgroups of the populations based on certain characteristics, to give them a better and more efficient treatment based on how they respond to the drug.

An example of a QSP based platform approach has been used to identify and predict alternative drug combinations of **opioid and non-opioid drugs to use as analgesics for the br**ain, with improved efficacy and reduced adverse effects.
This project combined together the expertise of various partners all over Europe and also from the USA, each with the competences – **a physiological-based pharmacokinetic model** to predict drug pharmacokinetics in human central nervous system was developed, combined with a target-binding kinetic model, a one based on experimental data in vivo on cellular signalling of analgesic related receptors, effectors and activation, and also a proper neural circuit model to quantify the drug effects on the relevant neuronal networks, based on preclinical and clinical data.
All of these elements together should allow us to predict the effect of a drug on a patient, depending on the way of administration, on the concentration, on the kinetics in vitro and in vivo, with a flow chart that is related to the individual features and characteristics

- as we can see in the image, with the same exact step, structure and process the same drug could have different effect, depending on the patient, and thanks to this approach we could try to model these differences.

Let's now make some examples of types of innovative molecules that we might be interested in screening during the development of innovative molecules

- **Kinases** – kinases and phosphatases, **with respectively phosphorylate and dephosphorylate proteins**, are involved in many processes of cellular activity and especially in the cascade of signal transduction; **their modification leads to an alteration of the protein substrate resulting in its modulation, and the kinases themselves can gain activity on phosphorylation**.

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By modulating one or more kinases we might obtain an useful therapeutic effect, especially since abnormalities in kinase activity are involved in various forms of cancer, diabetes and inflammatory diseases, rendering them an interesting pharmacological target.

So, which assay approaches could we use to screen a library of compounds searching for their activation or inhibitor activity on kinases?
We could also use techniques based on phospho-site specific antibodies, such as ELISA (which usually employs a colorimetric absorbance detection using AP or HRP but can also use fluorescent and luminescent substrates) and other assays (ex. immunoprecipitation), that can also be used in a cell-based format, which are usually more "physiological" and allow for an higher quality of information.
However, **it is also possible to detect the substrate deletion**: in fact **kinases always need to take the phosphate group from a source, which is ATP, and so we can detect its activity also monitoring the depletion of this molecule:** this is usually done employing the luciferase enzyme, which also needs ATP and can compete with the kinase for the same pool, **allowing us to see a lower signal when ATP is being consumed and so the kinase is active and viceversa – the signal is inversely proportional.**

- **INTRACELLULAR RECEPTORS** – another interesting target can be intracellular receptors, also called nuclear receptors even if not all of them are actually located in the nucleus but rather stay in the cytosol.
  They are usually bound and activated by a ligand, which causes a change in conformation, dimerization (either in homo or in heterodimers**) and then translocation into the nucleus**, where they can bind to specific responsive element and modulate the transcription of downstream gene.
  Usually therefore the ligand that binds them has an agonist function, but actually it is also possible that depending on its activity and on the dimerization too the result con actually be inhibitory.
  Intracellular receptors are involved in the regulation of genes correlated to a lot of physiological functions, we have steroid receptors in the cytoplasm, like glucocorticoid, androgen, estrogenic receptors that are active as homodimers, then RXR or retinoid X receptors, which instead are heterodimeric and include the vitamin D receptor and the retinoic acid receptor, and even orphan receptors, both dimeric and monomeric, for which ligands are unknown.
  Going into the screening assays used to obtain ligands that could have an effect and activity for these intracellular receptors, a very common system is to produce recombinant intracellular receptors ligand binding domains or NR-LBD, producing them with recombinant DNA technology in a His-tag for purification, and then using them to screen the ligand binding affinity usually employing a biotin-streptavidin interaction.

  For example this can be employed with a **RADIOLIGAND,** a streptavidin SPA bead, and a biotynilated NR-LBD, which attaches to the streptavidin SPA bead but is able to cause the scintillation only if the radioligand is able to attach to the receptor, getting close enough to the bead.

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We could also have **REPORTER-BASED ASSAY**, using always the ligand binging domain fused with a GAL4 binding protein, **so that once the receptor is activated by the ligand, it recognises the responsive element of GAL4** (which is composed of a series of UAS sequences and a TATA box) driving the downstream expression of a reporter gene, in this case luciferase.

GPCRs – another very interesting group of drug targets are G-protein coupled receptors, target of 40% of drugs in the actual market due to their involvement in a lot of physiophatological processes. **These receptors should actually be called 7 transmembrane receptors, since they have these transmembrane domains, while instead the G-protein was discovered as their first associated protein**, but is not the only one that exists and actually a lot of the transduction of these receptors is G-protein independent.
THE G-PROTEINS CONSISTS OF THREE SUBUNITS, alpha, beta and gamma, and they can couple to **the GPCR after ligand stimulation providing downstream effects**, with an auto-catalytic enzymatic activity that causes a change in conformation and determines the separation of the alpha subunit; however.

THEY ARE EFFECTORS ABLE TO BIND TO THE RECEPTOR AFTER ITS PHOSPHORYLATION, specifically the beta-arrestins 1 and 2, while arrestin 1 and 4 are visual and cone arrestin involved in the vision process; **after the binding of one of the beta-arrestins we have internalization of the receptor, and possibly its degradation and recycling, besides an uncoupling from the G-proteins** – in fact arrestins were named as such because their first observed effect was to stop G protein signalling, even if they have a lot more effects, like acting as scaffolds for the recruitment of other elements during signalling and leading to the phosphorylation of multiple targets.
Having cleared this, **GPCR engagement bay result in different signalling outputs, also by using an agonist** due to the fact that there are different active conformations that could be stabilised by an agonist, and depending on which one is activated different signals could transduce.

- For example two analgesics, morphine and oxycodone, have two different effects, the first one recruiting arrestins, while instead oxycodone triggers widespread receptor internalization: it is evident that GPCR have a very complex pharmacology, that can me modulated in various ways.
  Ligands for GPCRs therefore should be screened not only for their binding of course, but rather for the intrinsic activity that they explicit:
- we can have **FULL AGONISTS**, that explicit the maximum response possible at high concentration,
- also **PARTIAL AGONISTS**, that instead never reach the maximum effect and have half the potency of a full agonist at the same high concentrations after a certain point.
- Then we have **NEUTRAL AGONISTS**, that show no efficacy and they are basically antagonists, with the difference

- we can have **INVERSE AGONISTS**, that reduce the effect of a receptor that is normally constitutively expressed, like in the case of antihistamine used to treat allergies.

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If we are dealing with antagonists instead we need to test them against a certain level of agonist concentration, as we were saying, **and in this way we can also see if the antagonist is competitive or not**: specifically,

- if the curve **shifts to the right** at increasing concentrations reaching the maximum effect, we have a competitive inhibitor.
- if the curve goes **to the right but it does not reach the maximum efficiency it is a non-competitive antagonist**.

MOLECULAR SCREENING FOR LIGANDS OF GPCRS

One of the systems we could use is still radioligand binding, using a radioligand that binds to the receptor on the membrane and making it compete with our compounds to screen.

This gives us information about binding however, and not about the activity of the ligand, **but it can be useful to do a first screening to select only the ligands able to bind, and then among them do other screening to check for their activity**, whether it is an agonist, and antagonist, an allosteric ligand, etc.

Specifically, the activity on the GPCR can be measured at different levels:

- we could see it at the level of GTP hydrolysis, so the first event after the binding of the ligand to the enzyme, also using a radioactive heterogeneous assay;
- we could screen second messengers like cAMP (using fluorescence polarization, ELISA, the GloSensor, a modified and inactive luciferase that gets to work in the presence of cAMP due to a conformational change, or even HTRF) or Ca^{2+} which are produced during the signal transduction and are informative of the activity of the ligand
- we could also go even more downstream, screening the transcriptional activity derived from activation of GPCR, using reporter gene-based assays, and lastly we could observe the protein redistribution based on arrestins translocation or on the internalization of the receptor.
  - **SPECIFICALLY FOR ARRESTIN-DEPENDENT SIGNALING** we could investigate using BRET, attaching the donor on the GPCR and the acceptor on the beta-arrestin, so that the signal is present only when they interact.
    The beta-arrestin beings a protease able to cut the hinge in correspondence of a protease site, so if it interacts the transcription factor is freed and it goes to express a reporter protein.

LIGAND AND G-PROTEIN SELECTIVITY OF K-OPIOIDS RECEPTORS:

these receptors are highly desirable therapeutic targets to tret pain and addiction/affective disorders, **since they can be recognised by opioids but they do not cause addictive, hallucinogen and severe side effects like MOR opioids receptors**.

It has been observed that different agonists, both very active and with high potency**, actually bind in different ways to the receptor**, **stabilising different conformations**.

This seems to be due to different G-proteins, which act like allosteric modulators interacting and releasing from the receptor in a dynamic way.

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This was verified using BRET, with the donor attached to the G-protein alfa and the acceptor attached to the complex gamma-beta in figure, to observe the separation of the various subunits – depending of the subtype of G-protein present, like G_{i1}, G_{oA}, G_z, G_g a different modulation of the signal was observed starting from the same agonist, even with difference potency.

Coming from these observations, **some studies where made trying to develop more effective and safer analgesics, developing MOR agonists which should improve opioids safety and tolerability and be able to substitute morphine**.
The idea was to target the biased conformation of the receptor**, to trigger a specific type of downstream transduction**, like the activation of arrestines, **and therefore have a therapeutic effect with no or less adverse ones**.
While very interesting in principle however, al**l the structural diverse opioids still show morphine-like side effects**, like respiratory depression that can even lead to death.
An interesting example of this concept of biased agonism at MOR is TRV130, which should be able to activate the MO receptor causing a robust G-protein signalling, with potency and efficacy similar to morphine **but less involvement of the beta-arrestin pathway and less internalization of the receptor, leading in theory to the same analgesic effect but with less side effects**.

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