Course notes: IV. Drug design, Advanced therapy medicinal products and Microbiology

POSTGRADUATE EUROPEAN RADIOPHARMACY COURSE

Module I: Pharmacy

UNIVERSITY OF LJUBLJANA | FFA Faculty of Pharmacy

ETH
Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

univerzitetni klinični center ljubljana
University Medical Centre Ljubljana
Department for Nuclear Medicine

EANM EUROPEAN ASSOCIATION OF NUCLEAR MEDICINE

August 25th to September 5th 2025
Ljubljana, Slovenia

Publisher: University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, Ljubljana, Slovenia

Editors: Doroteja Novak, Petra Kolenc, Marko Krošelj

Title: Postgraduate European Radiopharmacy Course
Module 1: Pharmacy. Course Notes IV: Drug design, ATMP and microbiology in pharmacy (2025; Ljubljana)

Print: Tiskarna Skupina APIS d.o.o., 2025

25 copies

Price: 0 €

CONTENTS

Principles of medicinal chemistry

Prof. Dr. Tihomir Tomašič, University of Ljubljana, Faculty of Pharmacy, is a professor for the field of pharmaceutical chemistry at the Faculty of Pharmacy, University of Ljubljana. He is the lecturer of courses Pharmaceutical Chemistry I-III, Eutomers and Drug Design and Synthesis at the Uniform master's programme of Pharmacy. He is a member of the European Federation for Medicinal Chemistry (EFMC) and Slovenian Pharmaceutical Society. He was a visiting research fellow in the laboratory of Prof. Dr. Gerhard Klebe at the University of Marburg in Germany, at the company Xention Ltd. in UK and a postdoctoral fellow at the company Inte: Ligand GmbH in Austria, where he worked under the supervision of Prof. Dr. Sharon Bryant. He has a broad scientific network with many national/ international collaborations with researchers from USA, Hungary, Switzerland, Sweden, Netherlands, Italy, Lithuania, Argentina and Serbia. His research is focused on the discovery of bioactive molecules by computer-aided drug design methods, hit expansion and hit-to-lead optimization, especially in the field of antibacterial, anticancer and antiviral agents. He is a project leader of several national and bilateral project and is very active in COST actions (MuTaLig, Stratagem, Innogly).

The lecture has the following learning objectives:

1. Students are able to understand the basic principles of medicinal chemistry.

2. Students are able to assess the druggability of different organic compounds.

3. Students are able to recommend the possibilities for optimization of lead molecules.

4. Students are able to apply the methods of rational drug design to the field of radiopharmaceutical agents.

Summary of the lecture:

Medicinal chemistry, sometimes also named pharmaceutical chemistry, is one of the crucial fields of pharmacy dealing with the design, synthesis and biochemical evaluation of active ingredients and studies their action on molecular level. This definition is broad and at least in part overlaps with pharmacology, especially when discussing the molecular action of compounds and their metabolism. Especially in English speaking countries the term medicinal chemistry is often synonymous with drug design. The four-hour lecture is only capable of introducing this field of pharmacy to the listeners and will comprise of several topics in

medicinal chemistry, for detailed knowledge additional literature is suggested at the end of this presentation.

The idea for the new active ingredient starts with the decision for a validated target for a certain disease and the search of “hits”, a compounds with a certain affinity and desired activity on selected target relevant for a certain disease. The most important targets are biological membranes, proteins (enzymes, receptors, ion channels, etc.) and nucleic acids. Their structure and drugs that bind to these targets will be presented in some practical examples. Drug-target interactions that contribute to binding affinity will be presented together with approaches used in hit-to-lead and lead optimization stages, such as prodrugs, bioisosteres and design of mimetics (e.g. peptidomimetics, glycomimetics).

Discovering new compounds or in previous time herbal, animal and mineral material, which may improve individual’s health is as old as human history. The advances in organic synthesis, testing methods and later in other related scientific disciplines enabled fast growth in medicinal chemistry in last half of the century. Hits are generated by classical methods (screening/high throughput screening of natural products, synthetic compounds, observation of side effects of currently used drugs, drug repurposing, serendipitous discoveries, etc.) or by modern computer-aided drug discovery methods (structure- and/or ligand-based virtual screening, *de novo* design, fragment-based design, etc.). Hits are evaluated (Lipinski’s rule of five as a measure of drug-likeness will be presented and discussed) and with a bit of luck one or more lead compounds are discovered. Lead compounds are natural, semisynthetic or synthetic compounds with a certain pharmacological and biological activity, usually also with some undesired properties, that are a useful starting points for structural optimization.

Lead compounds are optimized to increase their activity and selectivity, reduce toxicity and also influence pharmacokinetic properties (bioavailability, predictable metabolism) of compounds. Pharmacophores should be identified, chemically reactive functional groups removed or replaced by less reactive moieties. Structures may be simplified especially in larger molecules, while in small molecules additional interactions with the active site may be achieved with further substituents. If the information about the binding conformation of a ligand is known, the increased rigidity of a derivative (cyclization, binding of sterically hindered functional groups) may yield a molecule with increased selectivity for certain type or subtype of target proteins. The binding of different moieties on the scaffold may influence the polarity of derivatives and the possibility to form interactions (ionic, H-bonds, nonpolar interactions…) with molecular target.

With the synthesis and evaluation of large number of structurally similar compounds on a certain target, the structure-activity relationship (SAR) may be stated. It defines the influence different substituents have on the biological activity of a compound. Quantitative structure-activity relationship (QSAR) is a process by which a biological activity of a molecule on specific target may be expressed as a mathematical equation in which the activity depends on various

physicochemical properties (lipophilicity, electronic properties and steric effects). Therefore, in a given system we may predict the activity of a derivative that has not yet been synthesized.

The principles currently described may be used in the design of new radiopharmaceutical agents. However, we have to take into account also the physicochemical properties of radionuclides. The possibilities and drawbacks in preparing selective technetium radiodiagnostics will be presented.

Take home messages:

• Be aware that the term medicinal chemistry is sometimes understood specifically for drug design and development.

• “Hits” and “lead compounds” only rarely possess appropriate physicochemical and pharmacological properties to be used as drugs. Different steps of lead optimization are needed to improve their properties and to understand the SAR of compounds.

• *In silico* methods in drug design facilitate the generation of “hits”, but every restrained used in the process reduces the chemical space.

• All the methods of drug design are also applicable to the field of radiopharmaceutical agents.

• You should always keep in mind the restrictions posed by the physical and chemical properties of an individual radionuclide.

References (further reading):

1. Lemke TL, Williams DA. Foye's principles of medicinal chemistry. 7th ed. Lippincott Williams & Wilkins, 2013.

2. Patrick GL. An introduction to medicinal chemistry. 6th ed. Oxford, UK: Oxford University Press; 2017.

3. AHFS Drug information 2019, American society of health-system pharmacists, USA, 2019.

4. Scientific journal: Nature Reviews Drug Discovery, Chemical Reviews, Journal of Medicinal Chemistry

5. Volkert WA, Hoffman TJ. Therapeutic radiopharmaceuticals. Chem Rev 1999, 99, 2269–2292.

6. Jurisson SS, Lydon JD. Potential technetium small molecule radiopharmaceuticals. Chem Rev 1999, 99, 2205–2218.

7. Willmann JK, van Bruggen N, Dinkelborg LM, Gambhir SS. Molecular imaging in drug development. Nat Rev Drug Disc 2008, 7, 591-607.

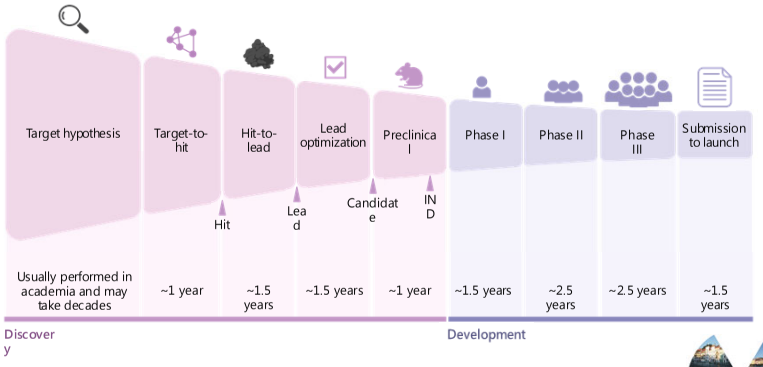
8. Chaturvedi S, Mishra AK, Small Molecule Radiopharmaceuticals – A Review of Current Approaches, Frontiers in Medicine 2016, 3, doi: 10.3389/fmed.2016.00005.

PRINCIPLES OF MEDICINAL CHEMISTRY

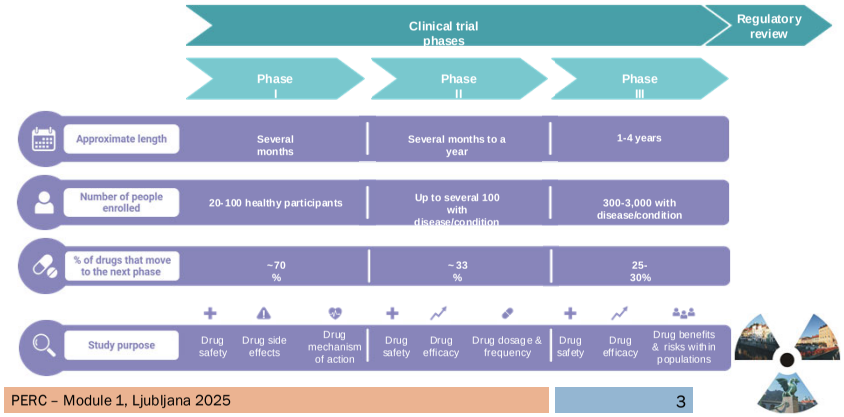
Prof. Dr. Tihomir Tomašič

University of Ljubljana, Faculty of Pharmacy

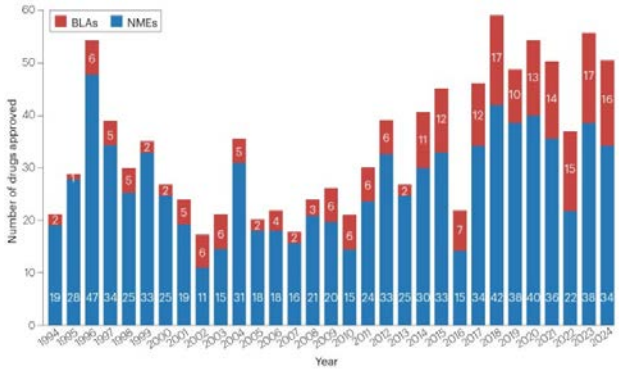
PHASES OF PRECLINICAL & CLINICAL DRUG DISCOVERY



PHASES OF CLINICAL TRIALS



NEW FDA DRUG APPROVALS



DRUG-TARGET INTERACTIONS

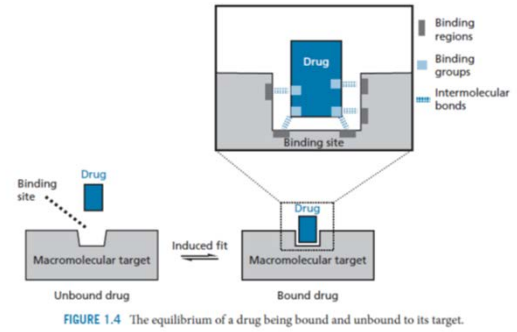


FIGURE 1.4 The equilibrium of a drug being bound and unbound to its target.

DRUG-TARGET INTERACTIONS

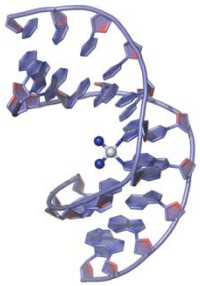
- $\Delta G° = \Delta H - T\Delta S$

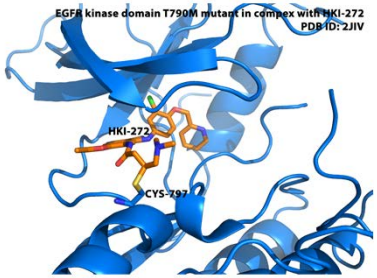
ΔH	-TΔS
covalent bonds	hydrophobic interactions
ionic bonds	loss of rotational and translational degrees of freedom
dipole interactions	
π-π, cation-π interactions	
hydrogen bonds	
Van der Waals interactions	

COVALENT BONDS

- strong (ΔG = 400 kJ/mol)
- irreversible
- nonselective?

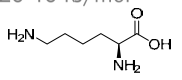
- drugs
  - DNA alkylating agents
  - organophosphates
  - penicillins
  - acetylsalicylic acid

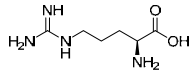


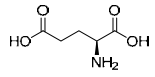


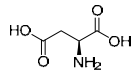
IONIC INTERACTION

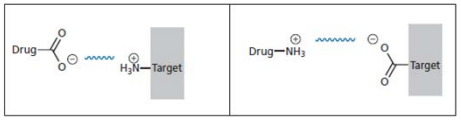
- 20-40 kJ/mol

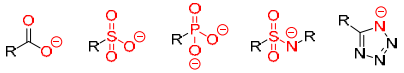

L-lysine

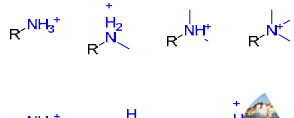

L-arginine

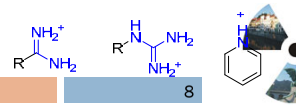

L-glutamic acid


L-aspartic acid









HYDROGEN BONDS (16-60 KJ/MOL)

FIGURE 1.6 Hydrogen bonding shown by a dashed line between a drug and a binding site (X, Y = oxygen or nitrogen; HBD = hydrogen bond donor, HBA = hydrogen bond acceptor).

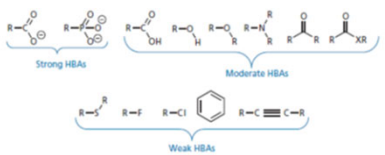


FIGURE 1.10 Relative strengths of hydrogen bond acceptors (HBAs).

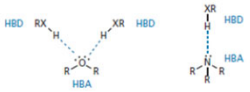


FIGURE 1.9 Oxygen and nitrogen acting as hydrogen bond acceptors (HBD = hydrogen bond donor, HBA = hydrogen bond acceptor).

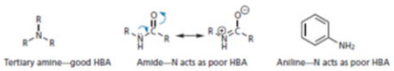
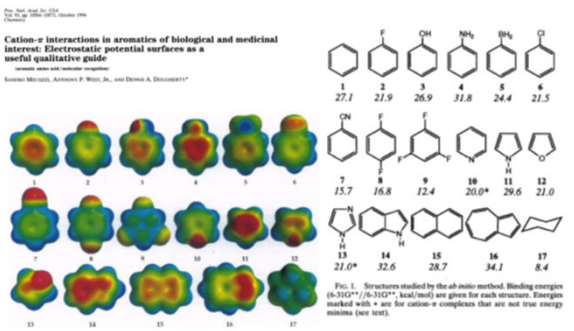


FIGURE 1.11 Comparison of different nitrogen containing functional groups as hydrogen bond acceptors (HBAs).

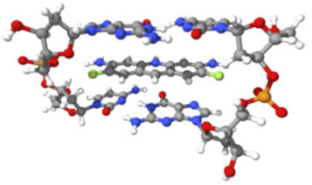
CATION-Π AND Π-Π INTERACTIONS



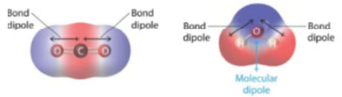
Cation–π interactions in aromatics of biological and medicinal interest: Electrostatic potential surfaces as a useful qualitative guide

FIG. 1. Structures studied by the *ab-initio* method. Binding energies (6-31G**//6-31G**, kcal/mol) are given for each structure. Energies marked with * are for cation–π complexes that are not true energy minima (see text).

intercalators



VDW, DIPOLE-DIPOLE AND ION-DIPOLE INTERACTIONS



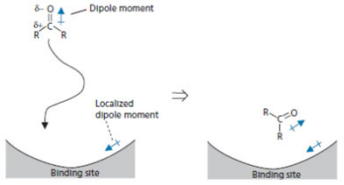


FIGURE 1.15 Dipole–dipole interactions between a drug and a binding site.

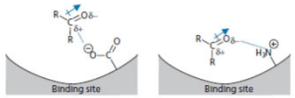


FIGURE 1.16 Ion–dipole interactions between a drug and a binding site.

2-4 kJ/mol

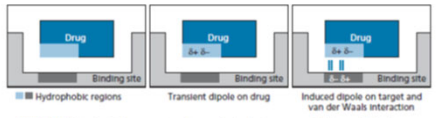


FIGURE 1.14 Van der Waals interactions between hydrophobic regions of a drug and a binding site.

HYDROPHOBIC INTERACTIONS (0.1-0.2 KJ/MOL Å^2)

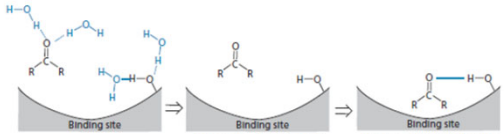


FIGURE 1.18 Desolvation of a drug and its target binding site prior to binding.

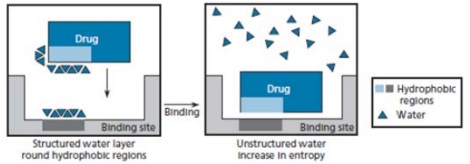
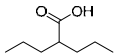
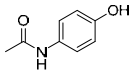
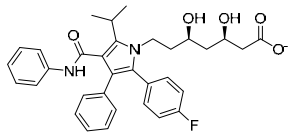


FIGURE 1.19 Hydrophobic interactions.

FUNCTIONAL GROUPS

- A specific group of atoms in a molecule, which has its own characteristic properties, regardless of the presence of the other atoms in the molecule

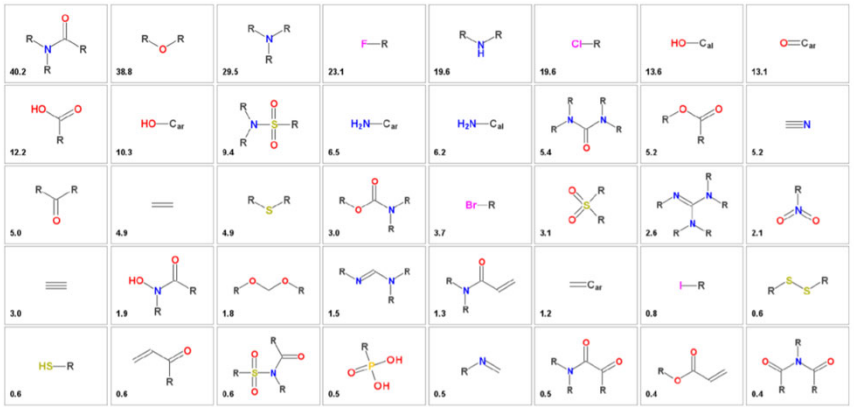
- Identify the functional groups in the structures of the following drugs:

ROLES OF THE FUNCTIONAL GROUPS

- influence on physico-chemical properties
- influence on the reactivity of the molecule
- influence on pharmacodynamics
- effect on pharmacokinetics
- impact on side effects and toxicity

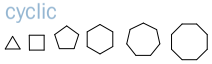
THE MOST COMMON FUNCTIONAL GROUPS IN BIOACTIVE COMPOUNDS



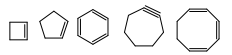
HYDROCARBONS

- composed of carbon and hydrogen

saturated (alkanes)
- linear
  ...CH_2-CH_2-CH_2...
- branched
  ... CH_2-CH-CH_2...
  CH_2-CH_2...
- cyclic



unsaturated
- alkenes
  ...CH_2CH=CH-CH_2...
- alkynes
  ...CH_2C≡C-CH_2...
- cyclic derivatives

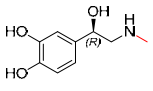


HYDROCARBONS

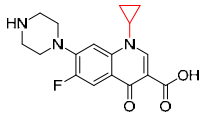
- very common functional groups in active substances
- they increase lipophilicity
- common sites of metabolic reactions

ALKANES

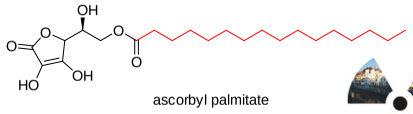
- functional group: **alkyl**
- prefix: alkyl-
- suffix: -ane



adrenaline



ciprofloxacin

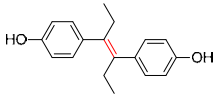


ascorbyl palmitate

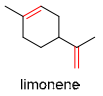
HYDROCARBONS

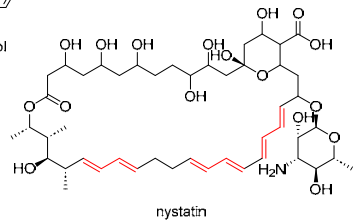
ALKENES

- functional group: **alkenyl**
- prefix: alkenyl-
- suffix: -ene
- *E/Z*

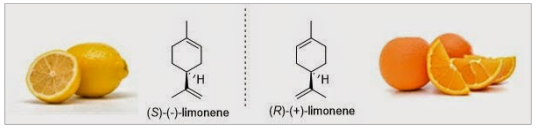


diethylstilbestrol


limonene



nystatin

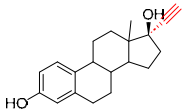


HYDROCARBONS

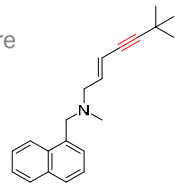
ALKYNES

- functional group: **alkynyl**
- prefix: alkynyl-
- suffix: -ine

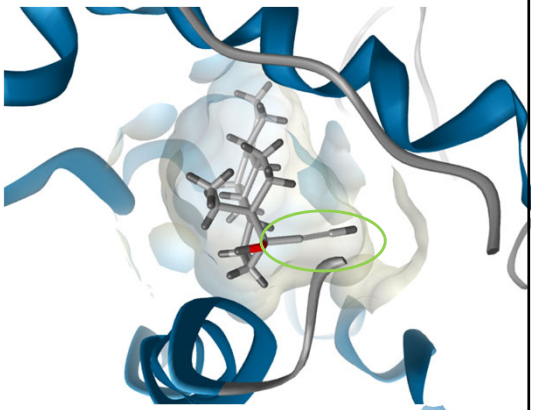
- linear structure



ethynylestradiol



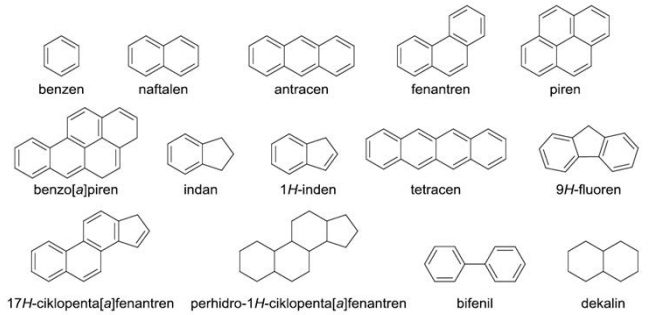
terbinafine



BENZENE AND CONDENSED AROMATIC HYDROCARBONS

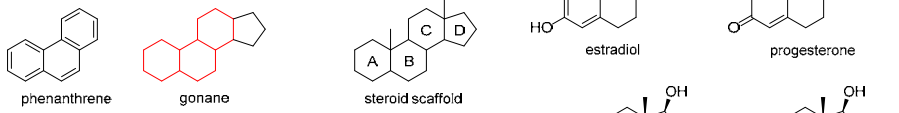
BENZENE

- functional group: **phenyl**
- very common

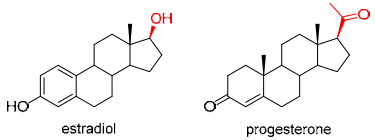


STEROIDS

- lipophilic
- structural element of the sex hormones

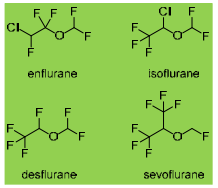


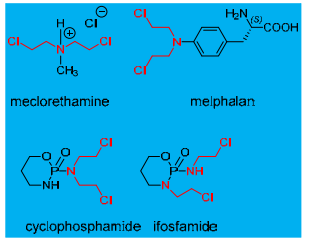
cyclopentanoperhydrophenanthrene
hexahydro-1*H*-cyclopenta[*a*]phenanthrene



HALOGENATED HYDROCARBONS

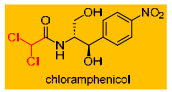
- lipophilic
- toxic – alkylating agents
- reactive





ALIPHATIC

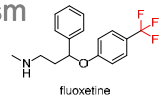
- inhalational anesthetics
- alkylating cytostatic agents
- antibacterial drug chloramphenicol

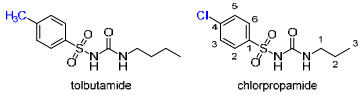


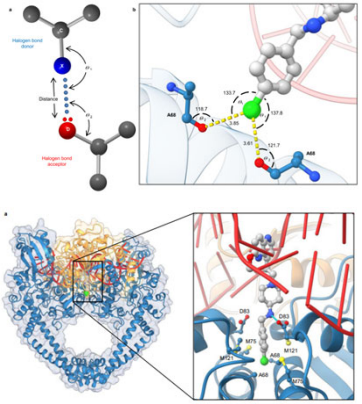
HALOGENATED HYDROCARBONS

AROMATIC

- formation of **hydrophobic** interactions
- formation of **halogen** bonds
- influence on **metabolism**



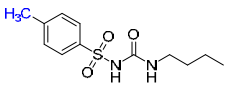


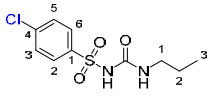


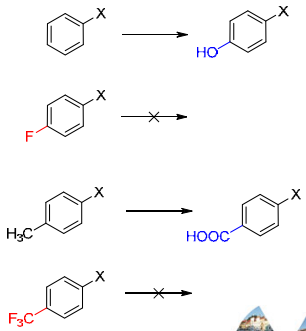
HALOGENATED HYDROCARBONS

AROMATIC

- influence on **metabolism**







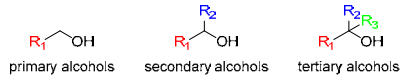
FUNCTIONAL GROUPS WITH OXYGEN

- alcohols
- ethers
- ketones
- aldehydes
- carboxylic acids
- esters

ALCOHOLS

- lipophilic and hydrophilic part
- formation of hydrogen bonds with other molecules and water
- effects on membranes and proteins

- functional group: **hydroxyl**
- prefix: hydroxy-
- suffix: -ol

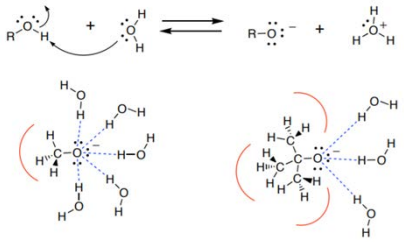


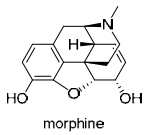
- increase of compound **polarity**
- formation of **hydrogen** bonds, **dipol-dipol** interactions

ALCOHOLS

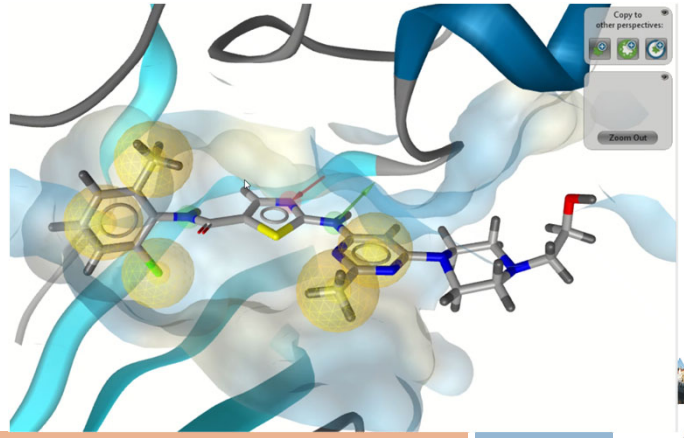
aliphatic alcohols

- increase in compound **solubility**
- additional interactions in the binding sites
- weak acids: pK_a ~ 16-18
- R group has important impact on acidity
- acidity is influenced by the solvation of the anion
- metabolism: phase 2 metabolic reactions



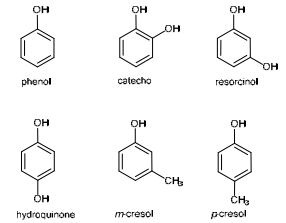


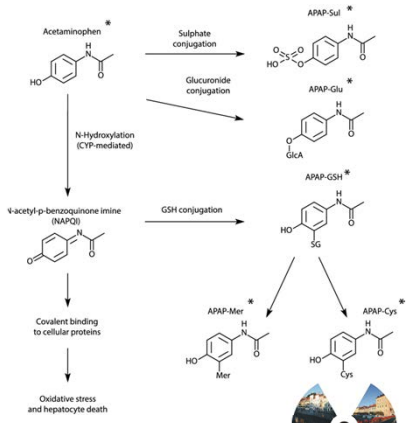
BINDING MODE OF DASATINIB IN ABL2/ARG KINASE



ALCOHOLS

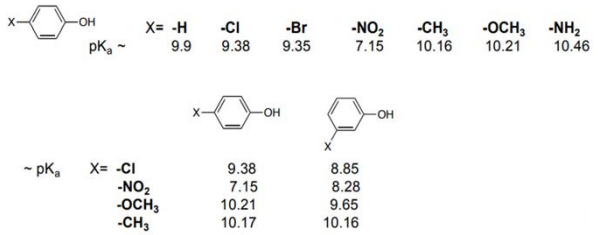
- aromatic alcohols = phenols
- more acidic than aliphatic alcohols
- oxidation to reactive metabolites
- antioxidative activity



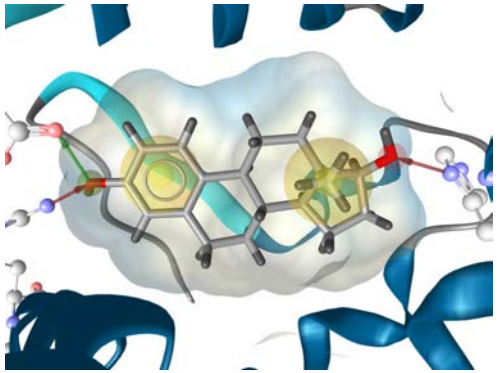


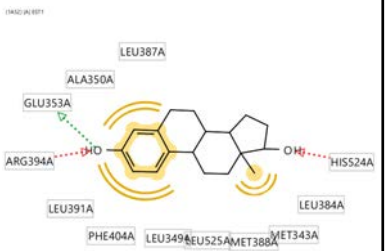
ALCOHOLS

- aromatic alcohols = phenols
- more acidic than aliphatic alcohols: pK_a highly dependent on the R group



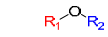
BINDING OF ESTRADIOL ON ESTROGEN RECEPTOR α



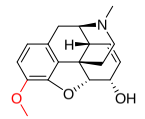


ETHERS

- a significantly lower possibility of forming H-bonds with water
- stable in alkaline conditions
- hydrolysis under strongly acidic conditions
- **lipophilic** derivatives
- chemically poorly reactive



symmetric – $R_1 = R_2$
non-symmetric – $R_1 \neq R_2$

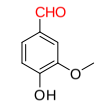


codeine

ALDEHYDES

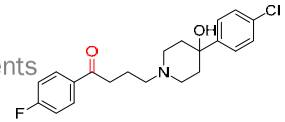
- contain a reactive **formyl** group
- soluble in organic solvents, methanal and ethanal also in water (H-bonds)
- aromatic aldehydes usually have a pleasant smell
- rapid oxidation to benzoic acids
- important starting compounds in the synthesis of active substances
- functional group: **aldehyde**
- prefix: formyl- or oxo-
- suffix: -al

- vanillin
- not toxic
- smell corrigens



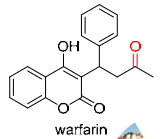
KETONES

- compounds with a carbonyl group
- less reactive than aldehydes
- acetone is miscible with water and non-polar solvents
- higher molecular weight - lipophilic compounds



haloperidol

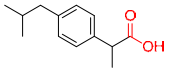
- functional group: **carbonyl**
- prefix: oxo-
- suffix: -one



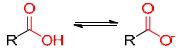
warfarin

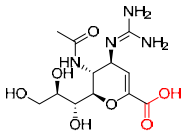
CARBOXYLIC ACIDS

- weaker than inorganic acids (pK_a ~ 5)
- equilibrium between **dissociated** and **undissociated** form
- they increase the polarity
- **salt** formation to increase solubility
- formation of strong ionic interactions and hydrogen bonds
- common in active substances



ibuprofen

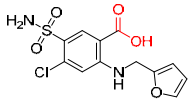




zanamivir

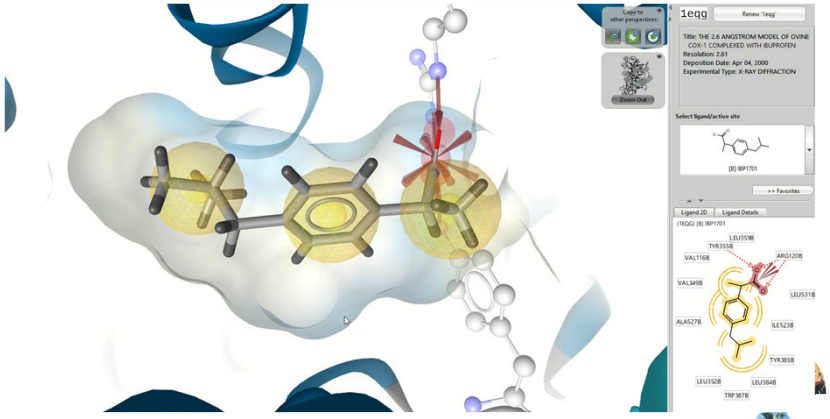
- functional group: **carboxyl**
- prefix: carboxy
- suffix: -ic acid

salicylic acid



furosemide

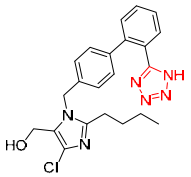
BINDING OF IBUPROFEN TO COX-1



CARBOXYLIC ACIDS

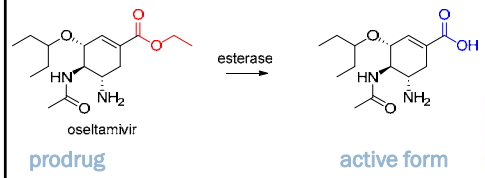
- bioisosteres are often used in design and optimization for improving pharmacokinetic properties
- **decreasing polarity** (increasing lipophilicity) to increase permeability

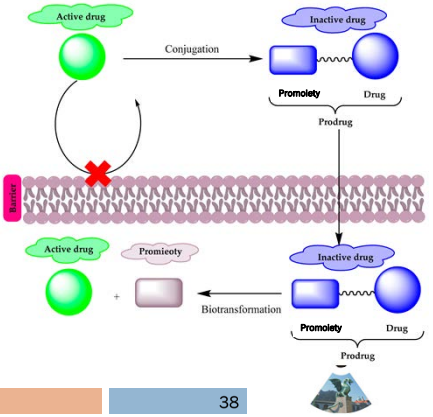
CO_2H $PO(OH)NH_2$	SO_2NHR $PO(OH)OEt$	SO_3H $CONHCN$
		



CARBOXYLIC ACIDS

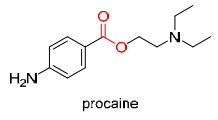
- formation of ester prodrugs

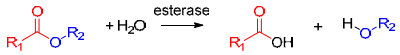




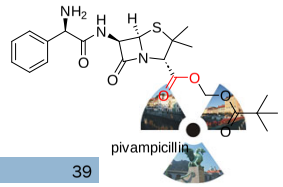
ESTERS

- derivatives of carboxylic acids and alcohols
- neutral
- more **lipophilic** than acid and alcohol
- rather **unstable** – carboxyesterases, cholinesterases, lipases





- functional group: carboalkoxy
- prefix: alkanoyloxy-, alkoxycarbonyl-
- suffix: alkylalkanoate

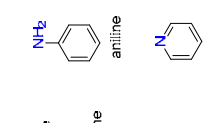


FUNCTIONAL GROUPS WITH NITROGEN

- amines
- imines
- nitriles
- amidines
- guanidines

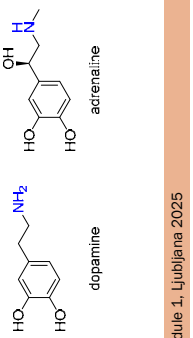
AMINES

- sp^3 hibridisation
- tetrahedral arrangement
- they are usually not chiral - pyramidal inversion
- basicity
- salt formation - **increase in solubility**
- equilibrium between the protonated and unprotonated form
- metabolism: oxidations, *N*-dealkylation
- effect of substitution in aromatic amines
- aliphatic amines are polar, aromatic more nonpolar
- aromatic amines are **toxic**



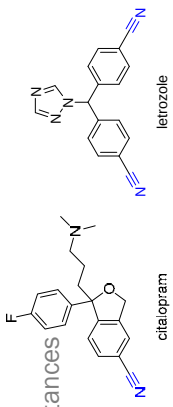
AMINES

- functional group: **amine**
- prefix: amino-
- suffix: -amine
- interactions: ionic interactions, hydrogen bonds, cation-π interactions, ion-dipol, dipol-dipol



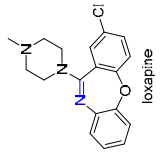
NITRILES

- not basic
- aromatic nitriles – present in active substances
- functional group: **nitrile**
- prefix: cyano-
- suffix: -nitrile or alkyl cyanide



IMINES

- **Schiff** base
- hydrolysis
- weakly basic
- functional group: **imine**
- prefix: imino-
- suffix: -imine

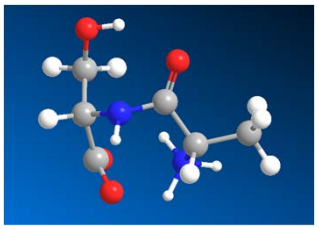


FUNCTIONAL GROUPS WITH OXYGEN AND NITROGEN

- amides
- nitro compounds
- carbamates
- ureas
- hydroxamic acids

AMIDES

- functional group: carboxamide
- prefix: carboxamido-, carbamoyl-
- suffix: -amide

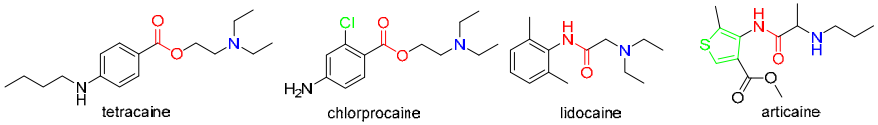


- chemically very weakly acidic or basic
- salts are unstable in an aqueous medium
- **tautomerism** and **planarity**

- in secondary amides, the oxygen of the carbonyl group and the hydrogen of the NH group are in the ***trans*** position
- simple synthesis

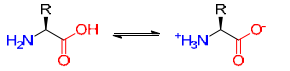
AMIDES

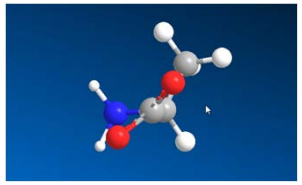
- more stable than esters
- example: local anesthetics

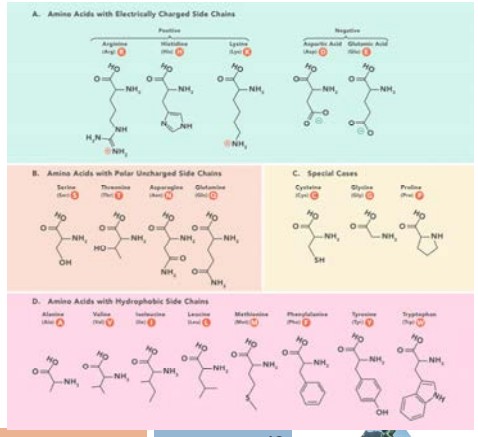


FUNCTIONAL GROUPS IN AMINO ACIDS

- 20 L-amino acids
- various physico-chemical properties

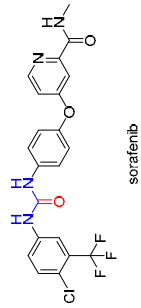


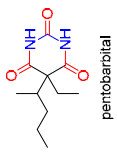


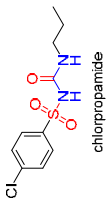


UREAS

- very weak bases
- rarely present in active substances
- more common are acylureas and sulphonylureas

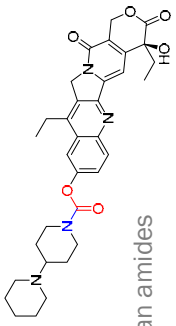


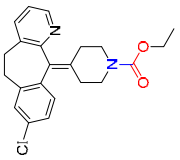


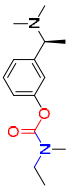


CARBAMATES

- esters of unstable carbamic acid
- slightly more polar than esters and amides
- hydrolysis in acidic and basic conditions
- more stable than esters, but less stable than amides
- important protecting groups in the synthesis

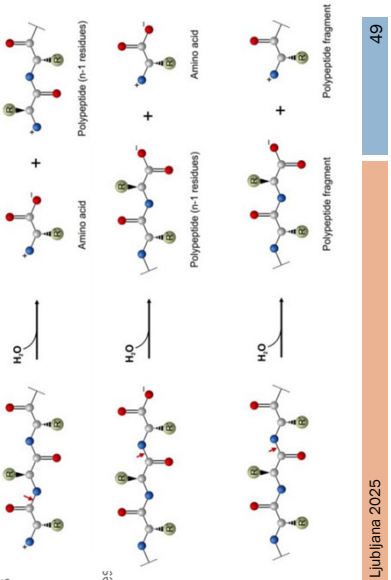






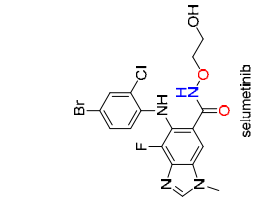
STABILITY OF PEPTIDES/PROTEINS

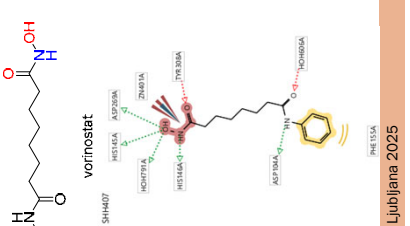
- hydrolysis of peptide bonds by peptidases
  - aminopeptidases
  - carboxypeptidases
  - endopeptidases



HYDROXAMIC ACIDS

- complexation of cations



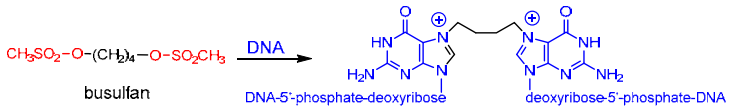


FUNCTIONAL GROUPS WITH SULPHUR

- thiols
- thioethers
- sulphoxides
- sulphones
- sulphonates
- sulphonamides

SULPHONATES

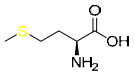
- esters of sulphonic acids
- more reactive than esters – rare in active substances

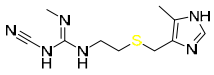


THIOETHERS

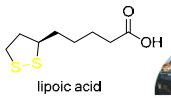
- also termed sulphides, sulphenes

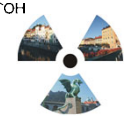
- sulphur analogues of ethers
- physico-chemical properties similar to ethers
- more reactive than ethers
- unpleasant odor


L-methionine


cimetidine

- disulphides
- formed by oxidation of thiols – reversible reaction

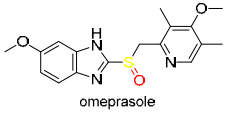

lipoic acid



SULPHOXIDES, SULPHONES

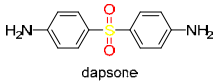
sulphoxides

- formed by oxidation of thioethers
- weakly basic properties


omeprasole

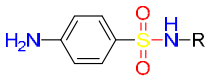
sulphones

- electron-withdrawing group
- weakly reactive


dapsone

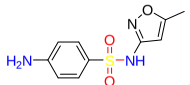
SULPHONAMIDES

- acidic
- soluble in alkaline solutions



R = H – pK_a = 10,4

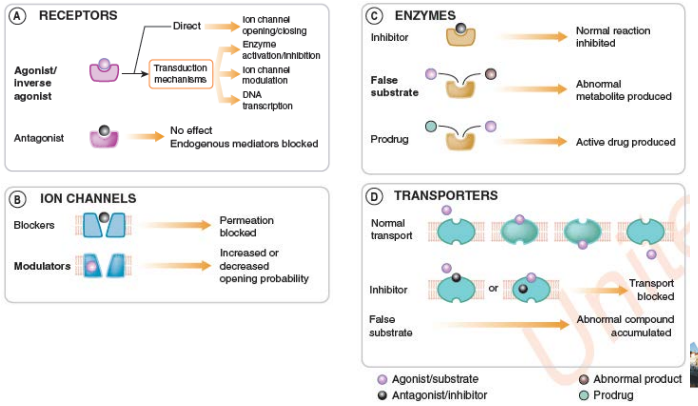
- R – usually a heterocycle
- effect on pK_a (5-7)
- effect on half-life



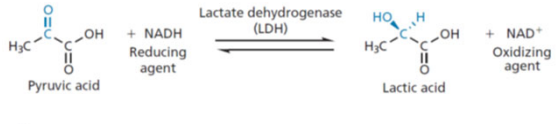
sulfamethoxazole pK_a = 5,0

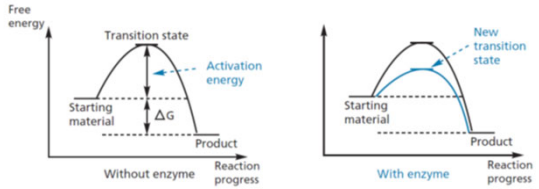
sulfadiazine pK_a = 6,5

TARGETS

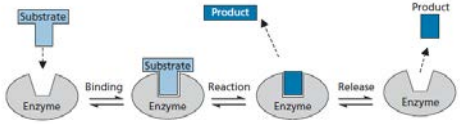


ENZYMES AS CATALYSTS





ENZYMES



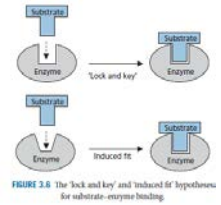


FIGURE 3.6 The 'lock and key' and 'induced fit' hypotheses for substrate–enzyme binding.

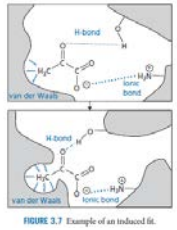
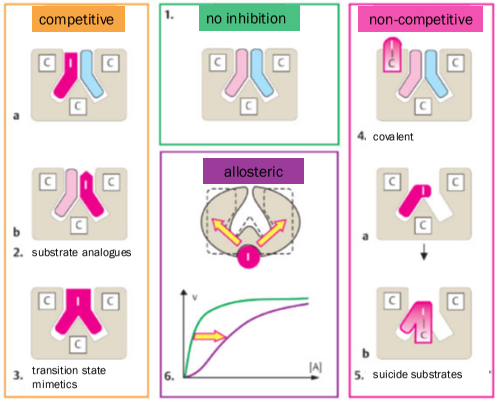


FIGURE 3.7 Example of an induced fit.

ENZYME INHIBITORS



ENZYME INHIBITORS

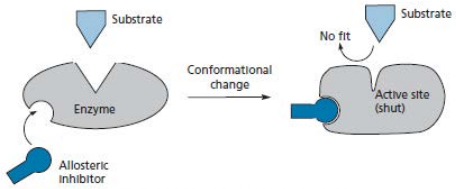
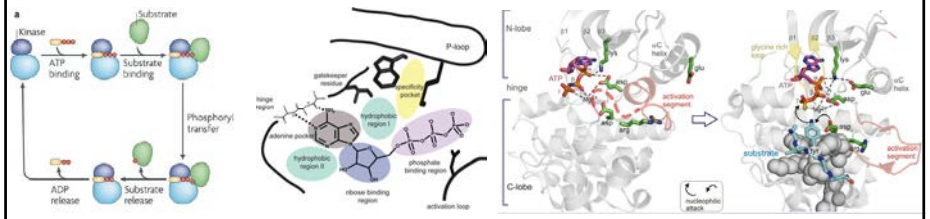
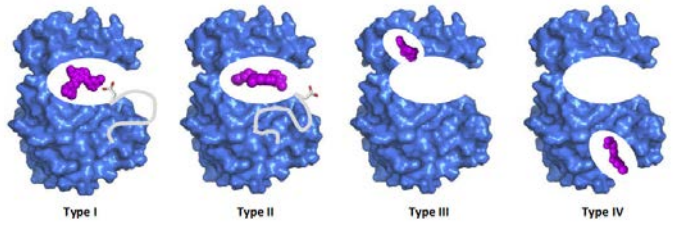


FIGURE 3.13 Allosteric inhibition of an enzyme.

PROTEIN KINASE INHIBITORS



PROTEIN KINASE INHIBITORS



RECEPTORS

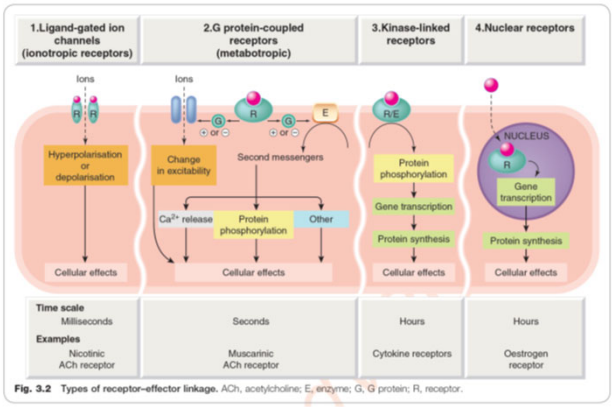
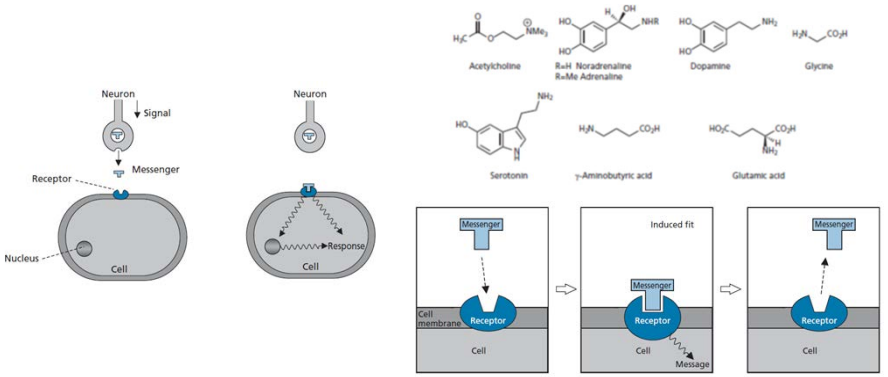


Fig. 3.2 Types of receptor–effector linkage. ACh, acetylcholine; E, enzyme; G, G protein; R, receptor.

RECEPTORS



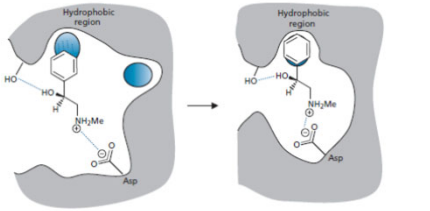


FIGURE 8.11 Binding of the natural chemical messenger resulting in an induced fit that activates the receptor.

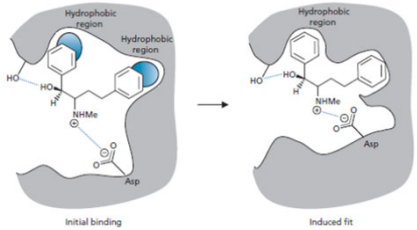


FIGURE 8.12 Binding of an antagonist leading to a different induced fit.

RECEPTORS

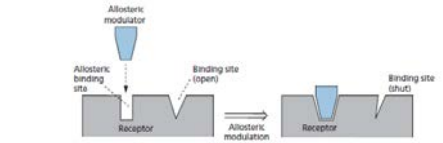


FIGURE 8.13 Principle by which an allosteric antagonist distorts a binding site.

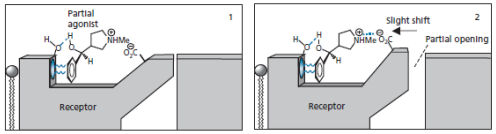
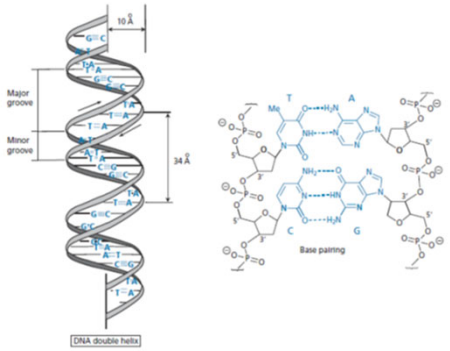
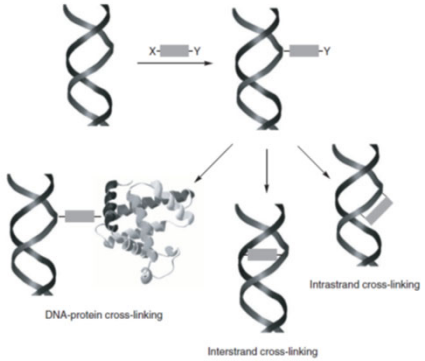


FIGURE 8.15 Partial agonism.

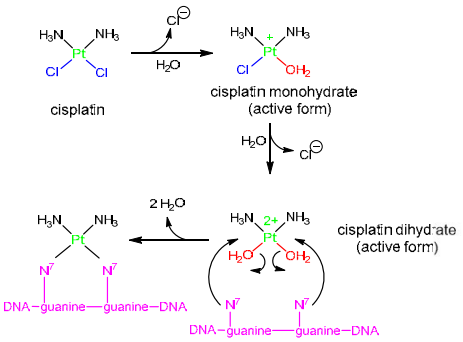
NUCLEIC ACIDS AS DRUG TARGETS

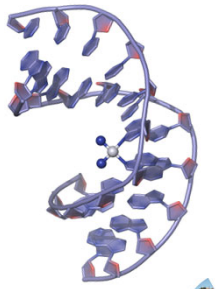


ALKYLATING AGENTS

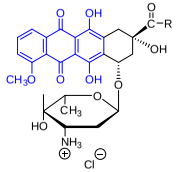


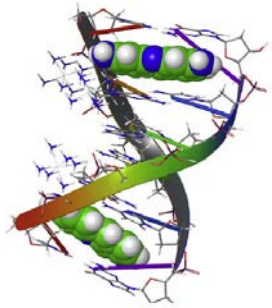
ALKYLATING AGENTS



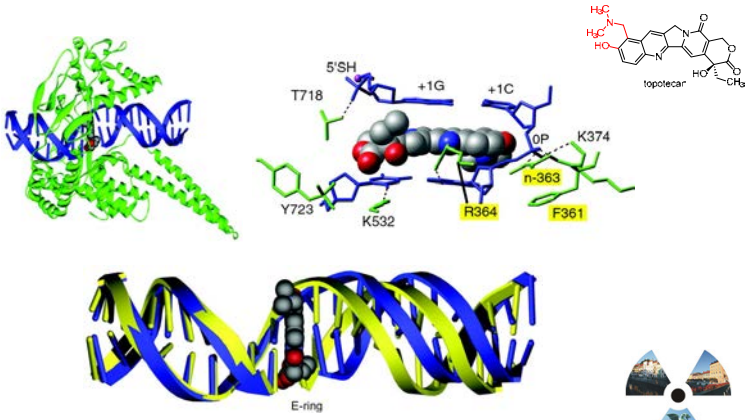


INTERCALATION





TOPOISOMERASE INHIBITORS



PRODRUGS

- inactive compounds
- (non)enzymatically converted in the body to the active drug
- 10% of all marketed drugs

- why?
  - improve absorption
    - improve membrane permeability by modifying lipophilicity
  - prolong drug activity
    - increase chemical/metabolic stability
  - improve patient acceptability
    - masking bad taste
    - decrease pain on the site of injection
  - target delivery
    - decrease toxicity/side-effects

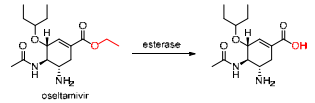
TYPES OF PRODRUGS

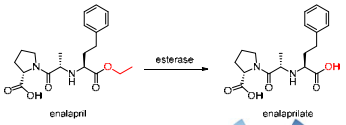
- carrier-linked prodrugs
  - temporary linkage of the active molecule (drug) with a transport moiety (promoiety)
  - active drug is a result of hydrolysis

- bioprecursor prodrugs
  - designed from a molecular modification of a drug
  - active drug is a result of a complex molecular transformation

CARRIER-LINKED PRODRUGS

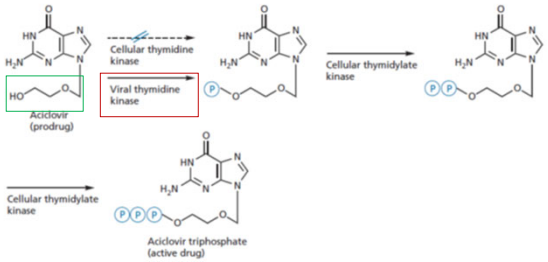
- prodrug is inactive/less active than the parent drug
- covalent bond between active drug and promoiety
- must be cleaved *in vivo*
- prodrug must be nontoxic
- promoiety/product of bond cleavage must be nontoxic
- effective conversion to the active drug after absorption
- high concentration of the active drug at the site of action





BIOPRECURSOR PRODRUGS

- no link to a carrier group
- prodrug is a molecular modification of the active drug
- active drug results from a more complex chemical /enzymatic conversion

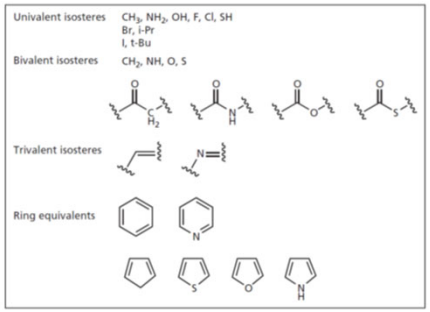


BIOISOSTERES

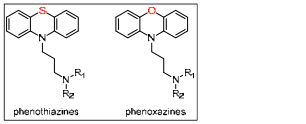
= atoms, groups, ions or molecules

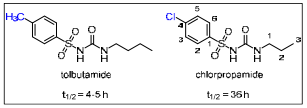
- similar volume
- similar shape
- similar valency
- same target

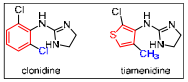
- why?

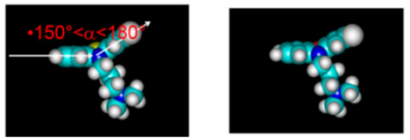


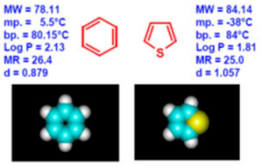
BIOISOSTERES











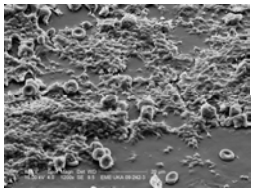
MIMETICS

- mimicry
- peptidomimetics – molecules that mimic the bioactive conformation of selected peptide

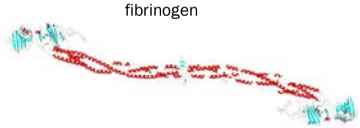
- glycomimetics
- mimetics of nucleic acids

PEPTIDOMIMETICS

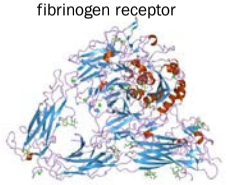
- platelet aggregation



fibrinogen



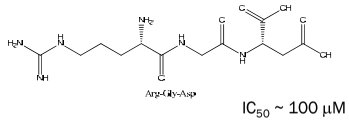
fibrinogen receptor



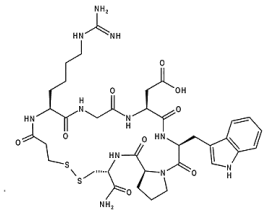
PEPTIDOMIMETICS

minimum AA sequence: RGD

Sistrurus miliarius barbouri
disintegrin – causes spontaneous bleeding



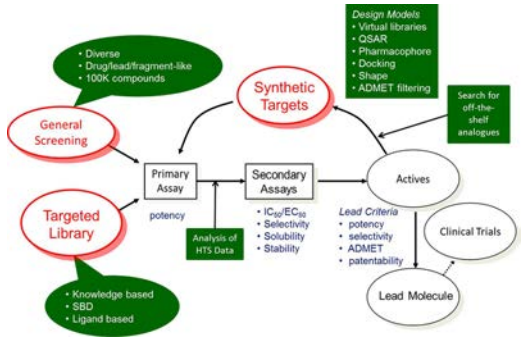
IC_{50} ~ 100 μM



eptifibatide
IC_{50} ~ 8.7 nM

conformational restriction

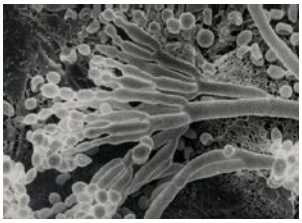
LEAD DISCOVERY

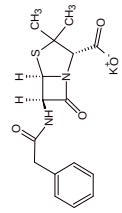


SOURCES OF NEW LEAD STRUCTURES

- Plant and microbial natural products
- Serendipitous discoveries
- Rational approaches (endogeneous transmitters)
- „Me-too“ research
- Chemogenomics
- Chemical biology
- Combinatorial chemistry / HTS
- Virtual screening
- Structure-based and computer-aided design
- Fragment-based design
- Combinatorial design

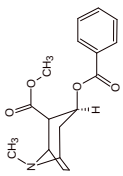
PRODUCTS FROM MICROBES





penicillin

PRODUCTS FROM PLANTS

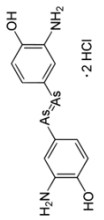


cocaine

HIGH-THROUGHPUT SCREENING

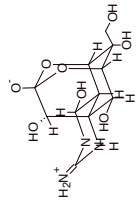
- High-throughput screening
- rapid screening of large number of compounds
- low hit rate
- design of compound libraries

Paul Ehrlich – screening of synthetic dyes for antibacterial activity



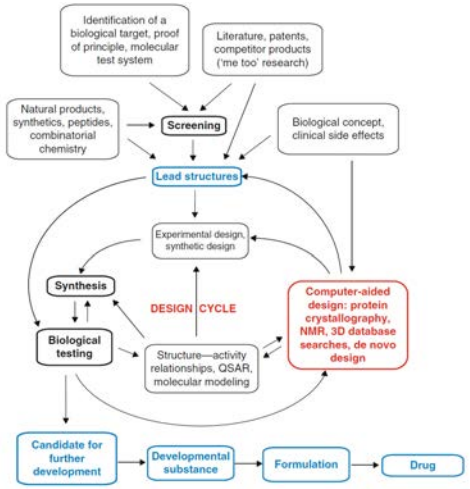
·2 HCl

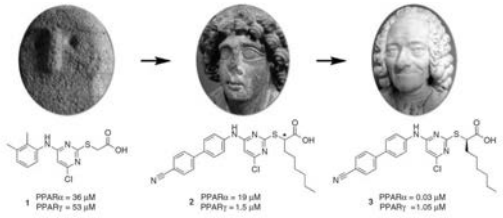
PRODUCTS FROM ANIMALS

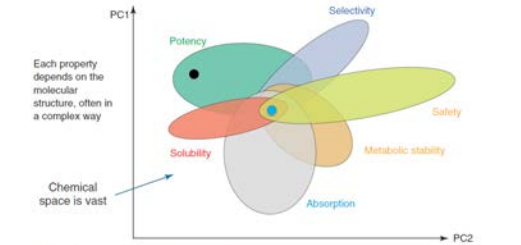


tetrodotoxin

RATIONAL DRUG DISCOVERY



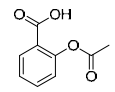




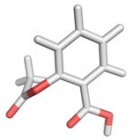
MOLECULAR GRAPHICS

- graphic representation of models of atoms and molecules, results of calculations
- standard atom colouring

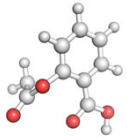
atom	colour
hydrogen	white
carbon	black/grey
oxygen	red
nitrogen	blue
sulphur	yellow
phosphorous	orange
fluorine, chlorine	green
bromine	brown
iodine	magenta



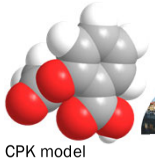
2D molecular graph



sticks model



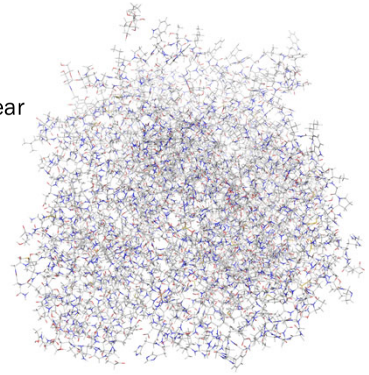
ball and sticks model



CPK model

PROTEIN STRUCTURE

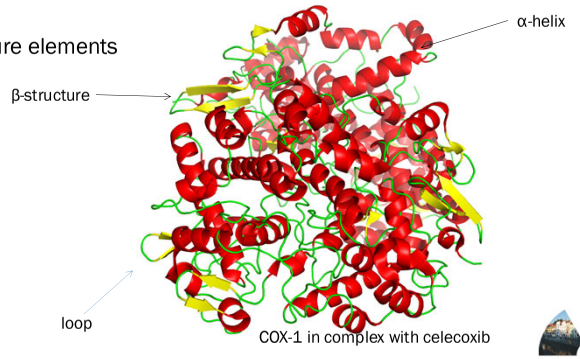
- visualisation of all atoms is not clear



COX-1 in complex with celecoxib

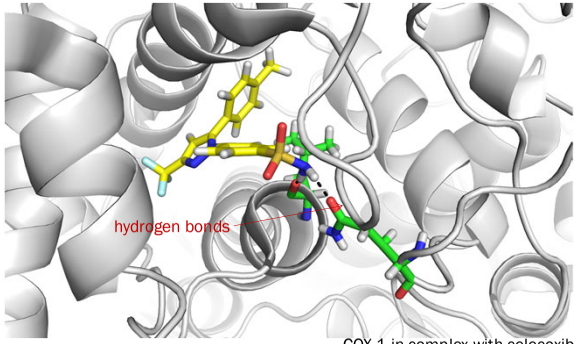
PROTEIN STRUCTURE

- secondary structure elements



COX-1 in complex with celecoxib

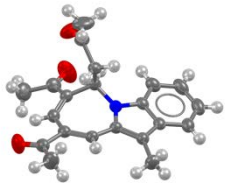
PROTEIN – LIGAND INTERACTIONS

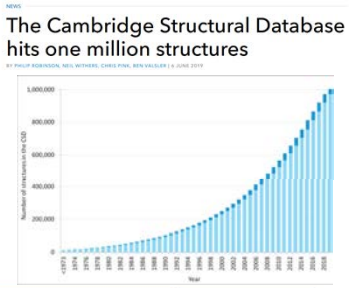


COX-1 in complex with celecoxib

EXPERIMENTAL DETERMINATION OF SMALL MOLECULE STRUCTURE

- Cambridge Structural Database (www.ccdc.cam.ac.uk)
  - X-ray diffraction analysis
  - neutron diffraction analysis



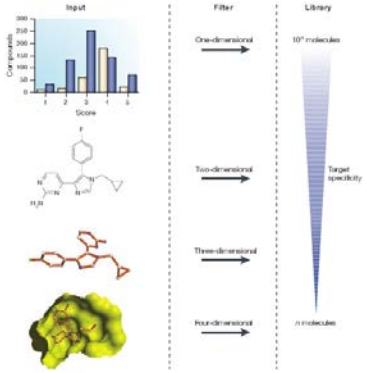


EXPERIMENTAL EVALUATION OF STRUCTURES OF MACROMOLECULES

- Protein Data Bank (www.rcsb.org)
  - X-ray crystallography
  - NMR spectroscopy
  - cryoEM

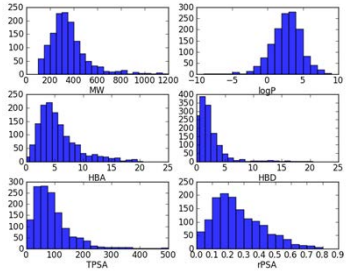
VIRTUAL SCREENING

- molecular descriptor filtering
- 2D ligand-based virtual screening
- 3D ligand-based virtual screening
- structure-based virtual screening



LIPINSKI RULES

- MW < 500
- -2 < logP < 5
- H-bond donors ≤ 5
- H-bond acceptors ≤ 10
- additional constraints:
  - Ar rings ≤ 5
  - number of rotatable bonds ≤ 5

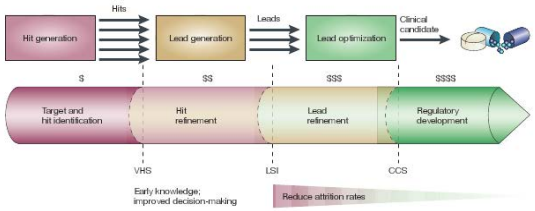


- drug-like molecules

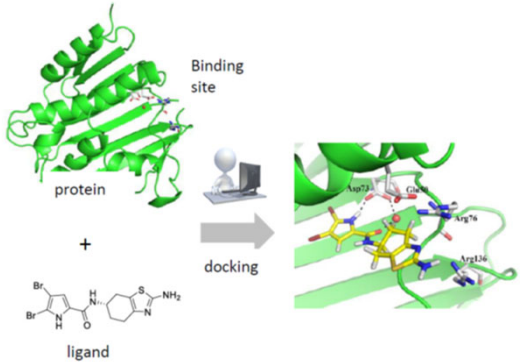
C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney
Adv. Drug Delivery Rev., 1997, 23(1-3), 3-25

LEAD-LIKE RULES

- during hit expansion and hit-to-lead optimisation MW, logP are usually increased
- lead-like rules:
  - MW < 450
  - -3,5 < logP < 4,5
  - H-bond donors ≤ 4
  - H-bond acceptors ≤ 10
  - Ar rings ≤ 4



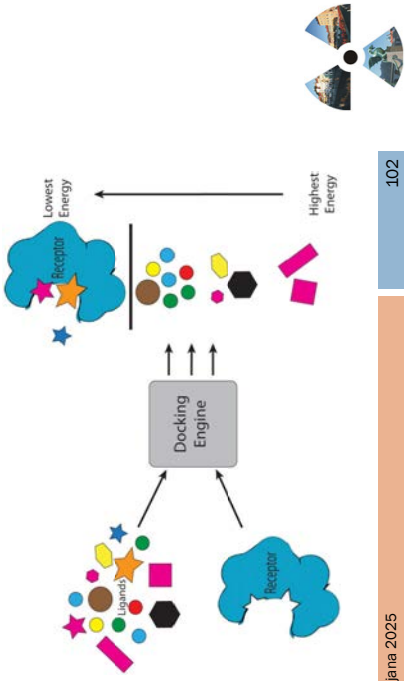
MOLECULAR DOCKING



MOLECULAR DOCKING

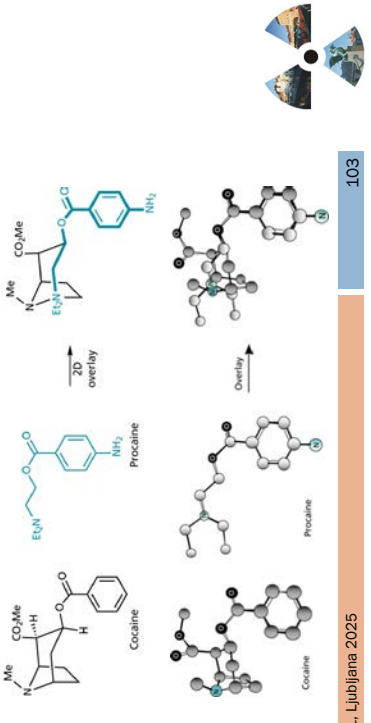
tetrodotoxin

STRUCTURE-BASED VIRTUAL SCREENING



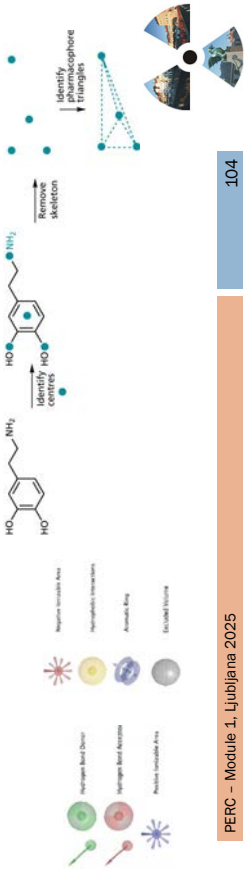
LIGAND-BASED SCREENING

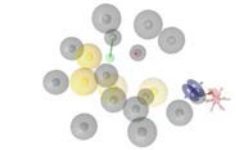
- molecules are 3D objects!



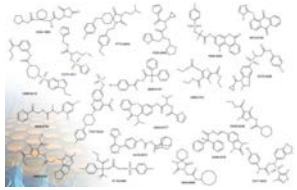
LIGAND-BASED VIRTUAL SCREENING

- pharmacophore modeling
- pharmacophore is an abstract description of molecular features that are necessary for molecular recognition of a ligand by a biological macromolecule

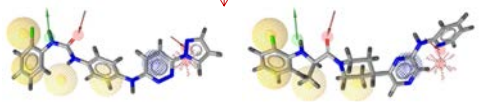




validated model for virtual screening



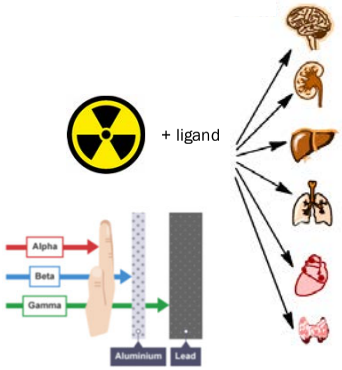
compound library



virtual screening hits

RADIOPHARMACEUTICAL AGENTS

- contain one or more radionuclides
- diagnostic radiopharmaceuticals
  - γ-particle emitting radionuclides
  - positron emitters
- therapeutic radiopharmaceuticals
  - β-particle emitting radionuclides
  - α-particle emitting radionuclides
  - low-energy electron emitters

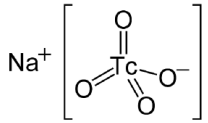


DIAGNOSTIC AGENTS

- most commonly used is ^{99m}Tc
- gamma emitter
- appropriate energy for detection: 140 keV
- $T_{1/2}$ = 6.02 h

- $^{99}Mo/^{99m}Tc$ generator – in the form of pertechnetate
- oxidation number: +7
- coordination number: 4

- stable, poorly reactive molecule



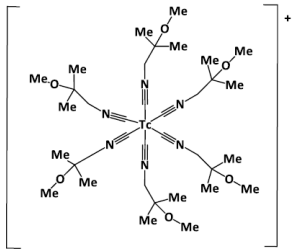
COMPLEX FORMATION

- coordination bonds between metal in electron donors
- commonly used electron donors for preparation of technetium compounds
  - amines ($R\text{-}NH_2$)
  - amides ($R\text{-}CO\text{-}NH_2$)
  - thiols (R-SH)
  - phosphines (R_3P)
  - oximes ($R_2C{=}NOH$)
  - isonitriles (RNC)

- technetium-essential radiopharmaceuticals
- technetium-tagged radiopharmaceuticals

TECHNETIUM-ESSENTIAL RADIOPHARMACEUTICALS

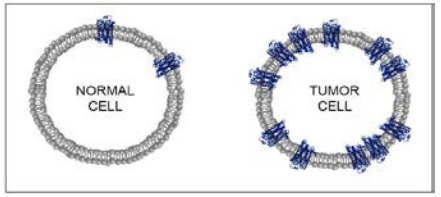
- myocardial perfusion scintigraphy



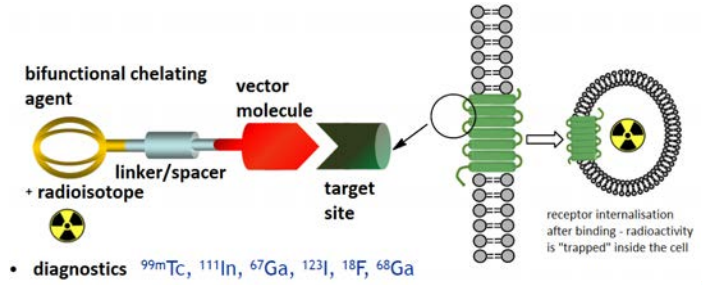
^{99m}Tc-sestamibi

TECHETIUM-TAGGED RADIOPHARMACEUTICALS

- radionuclide is bound to a ligand that is able to selectively recognise the target molecule/binding site
- attachment point does not affect the binding affinity
- tumour targeting

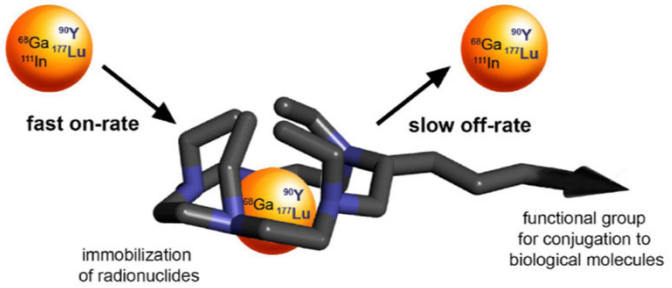


TUMOUR TARGETING

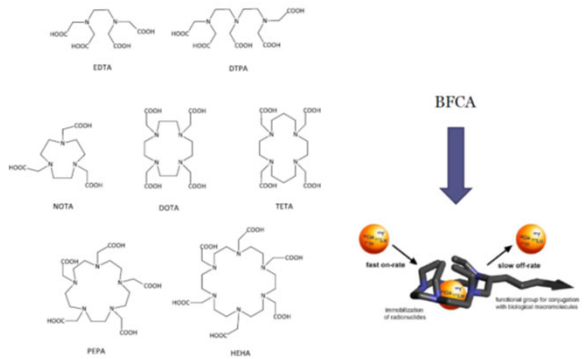


- diagnostics ^{99m}Tc, ^{111}In, ^{67}Ga, ^{123}I, ^{18}F, ^{68}Ga

BIFUNCTIONAL CHELATING AGENT



BIFUNCTIONAL CHELATING AGENT



RADIOPEPTIDES

- Target
  - somatostatin receptor (SSTR)
  - CCK2/gastrin receptor
  - folate receptor
  - integrin αvβ3
  - gastrin-releasing peptide receptor (GRPR)
  - glucagone-like peptide-1 receptor (GLP-1)
- Vector
  - somatostatin analogues or antagonists
  - minigastrin analogues
  - etarfolatide
  - Arg-Gly-Asp
  - bombesin antagonists
  - exendine analogues

SOMATOSTATIN

Somatostatin 14
Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
Octreotide
DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(ol), Sandostatin

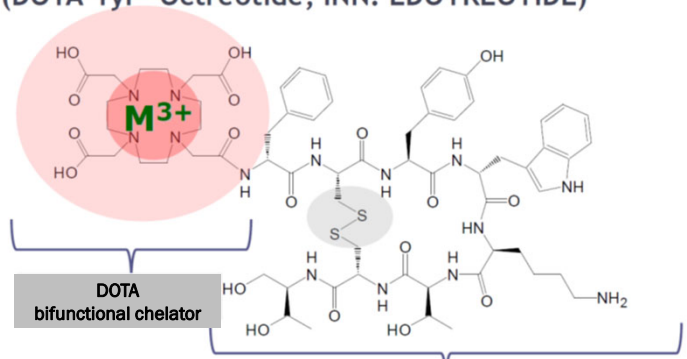
Pentetreotide, OctreoScan
DTPA-DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(ol)

DOTA-[Tyr³]octreotide (DOTA-TOC), Edotreotide: High affinity to SSTR2,5
DOTA-DPhe-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr(ol)

DOTA-[Tyr³]octreotate (DOTA-TATE): Higher affinity to SSTR2
DOTA-DPhe-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr-COOH

DOTA-[NaI³]octreotide (DOTA-NOC): Higher affinity to SSTR 3,5
DOTA-DPhe-Cys-NaI-DTrp-Lys-Thr-Cys-Thr-(ol)

(DOTA-Tyr³-Octreotide, INN: EDOTREOTIDE)



SUGGESTED READING

- Lemke TL, Williams DA. Foye's principles of medicinal chemistry. 7th ed. Lippincott Williams & Wilkins, 2013.
- Patrick GL. An introduction to medicinal chemistry. 6th ed. Oxford, UK: Oxford University Press; 2017.
- AHFS Drug information 2019, American society of health-system pharmacists, USA, 2019.
- Scientific journal: Nature Reviews Drug Discovery
- Volkert WA, Hoffman TJ. Therapeutic radiopharmaceuticals. Chem Rev 1999, 99, 2269–2292.
- Jurisson SS, Lydon JD. Potential technetium small molecule radiopharmaceuticals. Chem Rev 1999, 99, 2205–2218.
- Willmann JK, van Bruggen N, Dinkelborg LM, Gambhir SS. Molecular imaging in drug development. Nat Rev Drug Disc 2008, 7, 591-607.
- Chaturvedi S, Mishra AK, Small Molecule Radiopharmaceuticals – A Review of Current Approaches, Frontiers in Medicine 2016, 3, doi: 10.3389/fmed.2016.00005
- https://pharmacyce.unm.edu/nuclear_program/freelessonfiles/Vol12Lesson3.pdf

Radiolabelling of blood cells

Aljaž Sočan, PhD, MSc Pharm, University of Ljubljana, Faculty of Pharmacy, has more than 15 years of experience in radiopharmacy. He graduated at Faculty of Pharmacy, University Ljubljana where he also completed her postgraduate doctoral study in radiopharmacy. He started his career in 2004 as radiopharmacist at the Department of Nuclear Medicine at the University Medical Centre Ljubljana. In 2009, after completing defined programme of eductation he received the European Postgraduate Specialisation Certificate in Radiopharmacy, governed by European Association of Nuclear Medicine (EANM). In 2012 he succesfully finished Slovenian Specialisation in Radiopharmacy. Since 2013 he is head of Radiopharmacy at the Department of Nuclear Medicine at the University Medical Centre Ljubljana. For more than ten years he is active member of organising committee and a lecturer, covering pharmaceutical packaging and cell radiolabelling, on Postgraduate European Radiopharmacy Course, Module 1: Pharmacy, organised at Faculty of Pharmacy in cooperation with ETH, Zürich. He is involved in several national and international research projects. His research mainly focuses on work on stem cell radiolabelling and developement and aplication of new approaches on cell radiolabelling.

The lecture has the following learning objectives:

1. Students are able to understand role of blood cells and their biodistribution.

2. Students are able to explain and discriminate different radiolabelling approaches.

3. Students are able to recommend clinical applications of radiolabelled autologous cells.

4. Students are able to assess factors affecting cell radiolabelling and quality of radiolabelled cells.

5. Students are able to integrate requirements for radiolabelling and quality control of autologous cells.

Summary of the lecture:

Different nuclear medicine techniques allow non-invasive monitoring of the fate of autologous cells for medical research and the diagnosis of disease. Several blood cellular elements and other cells can be radiolabelled with different radionuclides and radiolabelling approaches for varies clinical applications (Table 1).

Regardless on the nature of the cells, radionuclide used or clinical application it is necessary to maintain both - cell viability and sterility and to avoid the operator's exposure to biological and radiation hazard during cell manipulation and radiolabelling. Manipulations and radiolabelling procedures of cells require strict aseptic conditions and should follow the

European Association of Nuclear Medicine (EANM) guidelines on current Good Radiopharmacy Practice (cGRPP) in the Preparation of Radiopharmaceuticals and Pharmacopoeial recommendations (1), European Pharmacopoeia (2) and local regulations. The use of an open or closed vial system on a laboratory workbench is not an alternative to a controlled aseptic environment. Cross contamination or mix-up of blood should be prevented at all times. Preparation of radiolabelled cells must be performed successive or by different people in different locations. All surfaces should be properly cleaned, decontaminated and disinfected prior to use, after all procedures are completed, and whenever surfaces are overtly contaminated. During radiolabelling approved written procedures should be followed at all times. All materials used should be identified and certified for human use. Whenever possible radiochemical purity should be checked and radiolabelling efficiency (percentage of radiolabelling of cells) calculated. Before release, radiolabelled blood should be checked for aggregation of clumping of cells and for presence of particulate contamination. At regular intervals the integrity of the cells should be ascertained by use of suitable procedures i.e. using trypan blue. Prior to administration control of patient identity should be performed (1- 4).

Table 1: Applicatons of radiolabelled blood cells in routine Nuclear Medicine clinical practice.

BLOOD CELLS (TYPE)	***RADIOLABEL***	***CLINICAL APPLICATION***
mixed leukocytes	^{111}In- and ^{99m}Tc-radiolabelled	non-quantitative imaging of infection and inflammation
Granulocytes or neutrophils	^{111}In- radiolabelled	quantitative imaging of infection and inflammation
lymphocytes	^{111}In-radiolabelled	lymphocyte migratory patterns and kinetic studies
platelets	^{111}In-radiolabelled	abnormal platelet deposition platelet kinetic and survival studies
red blood cells	^{99m}Tc-radiolabelled	GI bleeding, cardiac and vascular imaging
	^{51}Cr-radiolabelled	red cell survival studies, blood volume and red cell volume
heat-damaged red blood cells (spherocytes)	^{99m}Tc-radiolabelled	spleen imaging

RED BLOOD CELLS

Red blood cells (RBC) are the most common type of blood cell. Their main function is delivering oxygen from lungs to body tissues. Erythrocytes are continuously being produced in the red bone marrow of large bones. They develop from committed stem cells through reticulocytes to mature erythrocytes and live a total of about 120 days. The aging of erythrocyte undergoes changes in its plasma membrane making it susceptible to recognition by phagocytes and subsequently they are destroyed in the spleen, liver and bone marrow (5).

Radiolabelling with ^{99m}Tc

Radiolabelling approaches may be in vivo, in vitro and combined in vivo/in vitro. The basic mechanism of the radiolabelling of RBCs with ^{99m}Tc is in all approaches the same. The RBCs are "pre-tinned" by stannous ions for 10-30 min before ^{99m}Tc-pertechnetate is added to the cells. The stannous ions diffuse into the cell and get firmly bound to cellular components. The pertechnetate (^{99m}Tc) diffuses freely across the cell membrane and becomes reduced in the presence of stannous ions in the cell and subsequently binds to the beta chain of haemoglobin. At physiological pH stannous ions are likely to hydrolyse and precipitate and are rapidly removed from the blood by the reticuloendothelial system. To prevent hydrolysis and precipitation, stannous ions are thus usually in a complex of a weak chelator, such as pyrophosphate or medronate. The amount of stannous ions required for optimal radiolabelling is in the range of 10-20 µg per kilogram of body weight.

In vitro radiolabelling

In vitro radiolabelling of RBCs gives by far the highest labelling efficiency and the most stable labelling over time. It may be used for the determination of red cell and blood volume. It may also be employed in patients who are taking drugs, which may interfere or inhibit stannous ion transport through the cell membrane such as heparin, hydralazine etc, resulting in lower labelling efficiency (Table 2).

A small volume of anticoagulated blood (heparin or ACD) is incubated with an aliquot of a reconstituted stannous agent. Any excess of Sn^{2+} is oxidized by addition of 0.1% sodium hypochlorite and may be removed by centrifugation. The cells are separated and incubated with ^{99m}Tc-pertechnetate for 5-20 minutes with occasional mixing. After incubation unbound activity is washed away by addition of a few ml of saline and centrifugation. The cells are separated and re-suspended in saline before re-injection.

In vivo radiolabelling

This is the simplest and least time consuming radiolabelling technique. An injection of a reconstituted solution of stannous agent is followed by injection of ^{99m}Tc-pertechnetate 20-30 min later. The major disadvantage of this radiolabelling approach is a generally lower and more variable labelling efficiency. This may be due to insufficient Sn^{2+} incorporation into the cells which results in reduction of ^{99m}Tc-pertechnetate outside the RBC. Reduced ^{99m}Tc is then

not able to diffuse across the red cell membrane, resulting in a high background activity. Low labelling yields may also be a consequence of low haemoglobin concentration and/or low haematocrit.

In vivo/in vitro radiolabelling

More variations of in vivo/in vitro radiolabelling approach are in use. In all approaches the intravenous administration of a stannous agent is followed by withdrawal of an aliquot of pre-tinned blood 15-30 min after application. The excess of Sn^{2+} not incorporated into cells may be removed by centrifugation before ^{99m}Tc-pertechnetate is added to the cells. Alternatively blood may be taken into a shielded syringe containing an anticoagulant and the required amount of ^{99m}Tc-pertechnetate.The blood is then mixed with the ^{99m}Tc-pertechnetate and allowed to incubate for 5-20 min at room temperature with occasional mixing. The unbound activity is washed away by centrifugation before reinjection. Radiolabelled blood may alternatively also be re-injected without removal of unbound activity. With the later approach where no washing step is involved one can expect lower radiolabelling efficiency and higher background activity, depending on the complexity (number of washing steps avoided) of the procedure.

HEAT-DAMAGED RBC (spherocytes)

When radiolabelled RBCs are damaged by heat, they will be recognized by phagocytes and subsequently destroyed in the spleen, liver and bone marrow. This mechanism enables the use of heat-damaged ^{99m}Tc-RBCs for spleen imaging studies. Radiolabelled RBCs are damaged by incubation in water bath at 49.5 °C for 15 -20 min before reinjection. To sufficiently denature RBCs but preventing burst of RBCs, the temperature and incubation time are critical. Localization in liver indicates presence of cell destruction formatting bursting fragments.

Radiolabelling of RBCs can be affected by patient medication (Table 2). Drugs may interfere directly by reaction with Sn^{2+}, preventing accumulation of Sn^{2+} in the cells, or indirectly by affecting the RBC membrane or reduce the haematocrit and/or haemoglobin concentration. For more detailed information see (3, 6-8).

The usual administered activity of ^{99m}Tc-RBCs for adult patients is within the range of 500 – 1050 MBq. For children the activity may be adjusted according to body weight, with a minimum activity of 80 MBq in order to obtain images of sufficient quality (9). Only when in vivo radiolabelling approach is employed, breast feeding should be interrupted and the expressed feeds discarded due to the presence of free ^{99m}Tc-pertechnetate, which concentrates in the mammary gland. The total fractional activity ingested by the baby from in vivo radiolabelled RBCs is estimated to be 3 – 5 times higher than the activity in milk from in vitro radiolabelling. After in vitro radiolabelling breast feeding can be continued (10).

General recommendations for application of radioactive drugs are applicable for administration of radiolabelled RBC. Use of a teflon catheter or cannula should be avoided for administration of Sn^{2+} compounds, because Sn^{2+} can react with the catheter (11). To prevent

reaction of ^{99m}Tc-pertechnetate with traces of Sn^{2+} the stannous agent in the cannula and ^{99m}Tc-pertechnetate should not be given through the same system.

Radiolabelling with chromium-51 (^{51}Cr)

Radiolabelling of RBCs with chromium-51 is carried out in vitro: chromium-51 in the form of sodium chromate is incubated with whole blood containing ACD for approximately 10 -15 min. Chromate ion freely diffuses into the RBCs, where it is reduced to chromic ion (Cr^{3+}). Cr^{3+} bound to beta globin chain of the haemoglobin molecule is retained in the cell. The labelling process is stopped by adding ascorbic acid which reduces the chromate outside the cells to chromic ion. Alternatively free chromate is washed away by centrifugation.

Due to to limited number of Nuclear Medicine centers performing in-vitro radilabelling with chromium-51, production and availability of chromium-51 radiopharmaceuticals was discontinued in Europe in 2019.

NON IMAGING STUDIES

Basic principal in all non-imaging studies using radiolabelled RBC is the same: a known quantity of radiolabelled RBCs is added to an unknown volume of blood in the body. Radiolabelled blood is allowed to mix, and a known quantity of the mixture is then removed and quantitated (counted in a gamma counter). The ratio of the quantity measured after mixing (number of counts per mass) to the quantity added is the base for calculation of the unknown volume (3,12,13).

RBC mass and volume determination For RBC mass and volume determination one part of the radiolabelled blood is injected to the patient and the other part is used for a standard for further calculations. When complete mixing of re-injected radiolabelled blood has taken place (after 30 min or longer if the patient has splenomegaly), blood samples are taken for counting in a gamma counter. The red cell mass and plasma volume are calculated by using the dilution equations. The true blood volume is then obtained by summing up the red cell mass and the plasma volume. The total body haematocrit can be calculated by dividing the red cell mass by the true blood volume.

RBC survival

For RBC survival and sequestration studies ^{51}Cr-RBCs are reinjected to the patient and blood samples are obtained 3 times a week for 2 to 4 weeks after injection. To eliminate the need for decay correction the samples are counted at the end of the study. For the ^{51}Cr survival curve activity of the blood samples over time can be plotted on a semilog paper to linearize the curve. The patient's RBC effective half-life is calculated from the curve, the normal being 25 to 35 days. In case of a significant splenic sequestration of RBCs a significant and progressive increase in splenic uptake relative to the other expected site of RBCs, like blood pool or liver may be present. ^{51}Cr-RBC uptake is monitored using thyroid uptake probe positioning over spleen, liver and heart.

The usual administered activity of ^{51}Cr-RBC for RBC mass and volume determination is 0.8 – 1.5 MBq and 2.0 - 4.0 MBq for survival and sequestration studies.

LEUCOCYTES AND PLATELETS

White blood cells (WBC) or leukocytes are cells of the immune system defending the body against both infectious disease and foreign materials. Several different and diverse types of WBC exist. They are all produced and derived from a multipotent hematopoietic stem cell in the bone marrow. WBC are found throughout the body, including the blood and lymphatic system. One primary technique to classify WBC is to look for the presence of granules, which allows the differentiation of cells into two categories: granulocytes (neutrophils, basophils and eosinophils) and agranulocytes (lymphocytes, monocytes and macrophages) (5). Platelets or thrombocytes are a component of blood involved in the cellular mechanisms of primary haemostasis leading to the formation of blood clots. Platelets are not true cells. They gemmate from big leukocytes called megakaryocytes (5)

Platelets or thrombocytes are a component of blood involved in the cellular mechanisms of primary haemostasis leading to the formation of blood clots. Platelets are not true cells. They gemmate from big leukocytes called megakaryocytes (5)

Radiolabelling of WBC and platelets

Non-selective lipophilic ^{111}In and ^{99m}Tc complexes, which label all cells indiscriminately are used for radiolabelling of WBC and platelets. Generally radiolabelling efficiency is higher when ^{111}In complexes are used (80-90%) compare to ^{99m}Tc-HMPAO, where radiolabelling efficiency is normally 50-80%. Availability, low radiation exposure and better imaging characteristics are major advantages when ^{99m}Tc-HMPAO is used for radiolabelling. The clinical results using ^{111}In or ^{99m}Tc-HMPAO WBC are similar, but when ^{99m}Tc is used for radiolabelling, scans can be completed sooner, mostly with 1 h and 4-6 h imaging times. In-111-leukocytes are preferred for the investigation of inflammation in the kidneys (3,13,14).

Neutral lipophilic complexes rapidly diffuse across cell membranes and once inside the cell, the complex either dissociates and allows ^{111}In to bind to intracellular proteins (^{111}In-oxine or ^{111}In-tropolone) or breaks down to more hydrophilic complex which is unable to cross the cell membrane and is thus trapped in the cell (^{99m}Tc-HMPAO). In case of radiolabelling with ^{111}In-oxine, ^{111}In also binds to transferrin present in the plasma. Therefore the cells have to be washed thoroughly to remove plasma before radiolabelling. In case of radiolabelling with either ^{111}In-tropolone or ^{99m}Tc-HMPAO, radiolabelling can take place in the presence of small amounts of plasma. It is desirable to perform labelling of WBC in the presence of plasma in order to maintain normal cell function. Cells labelled in the absence of plasma show slower clearance through the lungs, indicative of loss of elasticity. To maintain normal function even 20% plasma is sufficient (14)

Because of non-selective approach, the cells need to be separated prior to radiolabelling. The separation of WBC and platelets from RBC in the blood can be achieved by sedimentation of anticoagulated blood. 30-50 ml of blood is taken into a 60 ml syringe containing an anti-

coagulant. The anti-coagulant of choice is acid-citrate-dextrose (ACD) in concentration 1.5 parts of ACD to 8.5 parts of whole blood. Erythrocyte sedimentation may be accelerated by the use of sedimentation agents such as 6% hydroxyethyl starch (Hespan). Alternatively 6% dextran or methylcellulose may be used. Normally 3 ml of Hespan per 30 ml of whole blood is used. Hespan may be added to ACD in syringe before blood is taken. Sedimentation time is generally 45-60 min and may be affected by different factors such as number of cells and certain disease states. Sedimentation may be carried out in the syringe placed upward in a suitable holder or in an Universal tube. After sedimentation the upper layer containing WBC and platelets is transferred into a sterile Universal tube. To separate WBC from platelets, the plasma is centrifuged at low speed e.g. 150 g. Platelet rich plasma is removed before radiolabelling agent is added to the pellet containing WBC and small amount of RBC. The mixture is incubated at room temperature. Incubation time varies depending on radiolabelling agent used. Generally it is longer when ^{99m}Tc-HMPAO is used. After radiolabelling the cells are re-suspended in saline or diluted plasma and free fraction of radiolabel is washed away by centrifugation. Radiolabelled cells are re-suspended before reinjection with saline or diluted plasma. Plasma is prepared from plated rich plasma by centrifugation at higher speed e.g. 500-700 g.

To ensure optimal viability of radiolabelled cells, the labelling should be completed as quickly as practical and the cells should be injected within 3 hours of removal.

Platelet reach plasma removed from the WBC may also be used for separation of platelets. This is achieved by additional centrifugation step at higher speed where platelets are centrifuged in the pellet and plasma can be removed. Radiolabelling procedure of the platelets is the same as for leucocytes.

The recommended dose for ^{111}In-WBC is 18.5 MBq and for ^{99m}Tc-HMPAO-WBC 400 MBq (range of 185-450 MBq). For children the activity may be adjusted according to body weight, with a minimum activity of 40 MBq of ^{99m}Tc-HMPAO-WBC in order to obtain images of sufficient quality (9). Breast-feeding should be interrupted and the expressed feeds discarded when ^{99m}Tc-HMPAO-WBC is used for imaging (10). Cell radiolabelling could be affected by a patient drug therapy (Table 2). For more detailed information see (3,6).

There is great interest in radiolabelling stem cells and imaging infection and inflammation using PET. Short half-life which does not allow sufficient time for imaging of leukocyte migration and monitoring of the fate of transplanted stem cells and stability of the radiolabelled cells are the main limitations for radiolabelling with positron emitters. Nevertheless ^{64}Cu with half-life of 12.7 h, which can react with a wide variety of chelator systems including DOTA, was reported to be a promising radiolabel (14,15).

Factors affecting radiolabelling

Many factors affect radiolabelling of cells. They can result from patient him / herself or can be introduced by operator / facilities used for radiolabelling. The most frequently radiolabelling efficiency is affected by presence of disease in patient and / or patient's medication (Table 2). Other factors, which may affect the labelling efficiency are plasma concentration, types of cell collected, method of radiolabelling, choice of sedimentation agent, choice of anticoagulant,

stability of cell chelator, concentration of ligand and radionuclide, concentration and number of cells and volume of ingredients, pH, temperature, cell damage (needle for blood collection and transfer, transport, manipulations...) and poor operator technique or inter-variability. Low labelling yields for RBC labelling have been reported when stannous agent is administered through a Teflon cannula (3,4).

Radiolabelling efficiency

The percentage of radioactivity incorporated into the cells is usually described as radiolabelling efficiency. It should be determined as the last step before radiolabelled cells are re-suspended and re-injected: the radiolabelled cells are separated from the labelling medium by centrifugation. The activity of the labelled cells is measured before and after separation. Labelling efficiency is then calculated:

$$\%\ Radiolabelling\ efficiency = \frac{radioactivity\ on\ separated\ cells}{total\ activity\ before\ separation} \times 100\%$$

Nevertheless a high radiolabelling efficiency is not necessarily indicative of a good labelling procedure or viable cells. Regardless on the radiolabelling efficiency it is important to obtain a viable population of cells for reinjection.

Cell viability

It is essential that radiolabelled cells remain viability when they are re-injected to the body. They may be damaged from the harvesting and/or radiolabelling procedures. Cell viability is most frequently assessed by Trypan blue exclusion assay. Trypan blue is a stain which is incorporated into dead cells, whereas live cells are not coloured. Equal volumes (0.1 – 0.2 ml) of radiolabelled cell suspension and 0.4 - 0.5% Trypan blue are gently mixed in appropriate tube and incubated at room temperature for 5 minutes. The mixture is applied on a haemocytometer slide and cells counted under a light microscope. The percentage of viable cells is the number of viable cells divided by the number of dead and viable cells. Usually it should be >95%.

Table 2: Drugs affecting radiolabelling of blood cells

White blood cells		**Red blood cells**	
calcuim channel blockers(nifedipine)	agranulocytosis	hydralazine	oxidation of stannous ions
captopril	bone marrow suppresion	methyldopa	oxidation of stannous ions

sodium valproate	bone marrow suppresion	calcium channel blockers	nterfering with stannous ion, decreased uptake
metronidazole	leukopenia	cyclosporin	Interfered incorporation into the cell
allopurinol	leukopenia	propranolol	decreased labelling efficiency
prednisolone	adherence to nylon	chlorothiazide	decreased labelling efficiency
alcohol	adherence to nylon	furosemide	decreased labelling efficiency
Aspirin	adherence to nylon	digoxin	decreased labelling efficiency
lignocaine	agranulocytosis	prazosin	decreased labelling efficiency
Ibuprofen	agranulocytosis	adriamycin	decreased labelling efficiency
adriamycin	agranulocytosis	sulphasalazine	changes in cell membrane
cyclophosphamide	agranulocytosis	cephalosporins	changes in cell membrane
vincristine	agranulocytosis	omeprazole	changes in cell membrane
azathioprine	agranulocytosis	quinine	changes in cell membrane

Take home messages:

• Be aware of dangers of working with blood samples to personnel o Hepatitis B vaccination (local regulations) o Needle stick policy

• Maintain both - cell viability and sterility and avoid the operator's exposure to biological and radiation hazard during cell manipulation and radiolabelling.

• Work in aseptic conditions following the EANM guidelines on current Good Radiopharmacy Practice (cGRPP) in the Preparation of Radiopharmaceuticals and PhEur, Monograph 5.19 Extemporaneous preparation of radiopharmaceutical preparations[[.

• The use of an open or closed vial system on a laboratory workbench is not an alternative to a controlled aseptic environment.

• Cross contamination or mix-up of blood should be prevented at all times.

• Preparation of radiolabelled cells must be performed successive or by different people in different locations.

• Avoid the use of needles where/whenever possible

• Have a proper waste disposal policy

References (further reading):

• Guidelines on current good radiopharmacy practice (cGRPP) in the preparation of radiopharmaceuticals. (http://www.eanm.org/scientific_info/guidelines/)

• The European Pharmacopoeia. Monograph 5.19. Extemporaneous preparation of radiopharmaceutical preparations.

• Ellis BL, Sampson CB. Radiolabelling of blood cells - theory and practice. In: Sampson CB, editor. Textbook of radiopharmacy theory and practice. 3rd ed. Amsterdam: Gordon and Breach Science Publishers; 1999; p. 83-104.

• Solanki KK and Dondi D. Radiolabelled autologous cells: methods and standardization for clinical use. IAEA, 2015

• Aberts B et al.Molecular biology of the cell, 5th ed., 2008

• Sampson CB. Complications and difficulties in radiolabelling blood cells: a review. Nucl Med Commun 1996;17:648-58.

• Sampson CB. Adverse reactions and drug interactions with radiopharmaceuticals. Drug Safety 1993;8:280-94.

• Lee HB, Wexler JP, Scharf SC, Blaufox MD. Pharmacologic alterations in Tc-99m binding by red blood cells: concise communication. J Nucl Med 1983;24:397-401.

• Lassmann M, Biassoni L, Monsieurs M, Franzius C, Jacobs F.The new EANM paediatric dosage card. Eur J Nucl Med MolImaging 2007;34:796-8.

• International Atomic Energy Agency. Applying radiation safety standards in nuclear medicine No. 40. IAEA safety related publications, Appendix IV: Cessation of breast feeding. Vienna: IAEA; 2005. p. 97-9.

• Millar AM, Wathen CG, Muir AL. Failure in labelling of red blood cells with 99mTc: interaction between intravenous cannulae and stannous pyrophosphate. Eur J Nucl Med 1983;8:502-4.

• Karlson KE and Senn LaV Y. Simultaneous Determination of Red Cell Mass and Plasma Volume with Cr51 and I131 Using a Pulse Height Analyzer.Ann Surg. 1963 August; 158(2): 309-318.

• Wilson MA. Textbook of nuclear medicine. Philadelphia: Lippincott-Raven Publishers; 1998

• Ballinger JR, Gnanasegaran G. Radiolabelled leukocytes for imaging and inflammation: how radiochemistry affects clinical use. QJ Nucl Med Mol Imaging 2005;49:308-18.

• Bhargava KK et al. In-vitro human leukocyte labelling with 64Cu: An intraindividual comparison with 111In-oxine and 18F-FDG. Nucl Med Biol 2009;36(5):545-549.

RADIOLABELLING OF BLOOD CELLS

ALJAŽ SOČAN, MPharm, PhD
Universitiy Medical Centre, Ljubljana
Department for Nuclear Medicine

CONTENT

Vascular system

Blood Cells

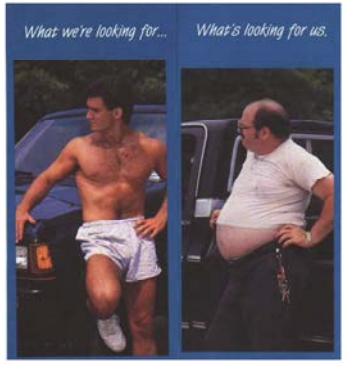
Clinical indications

Radiolabelling of blood cells
- Radioactive isotopes
- Approaches

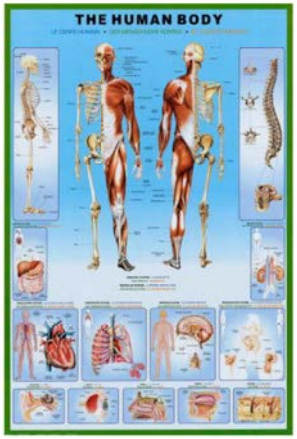
Factors affecting radiolabelling

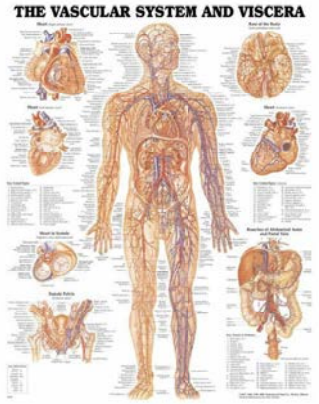
- Practical demonstrations (WBC, RBC radiolabelling)

HUMAN BODY



- 56% water
- Bones
- Muscles
- Organs
- Body fluids



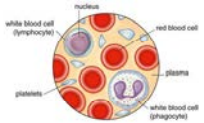


BLOOD

Suspension of cells in plasma

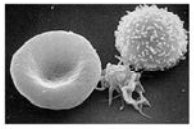
(92% water, electorlites, blood plasam proteins (albumin, globulin, fibrinogen), hormones)

- Erytrocytes (red blood cells)
- Leucocytes (white blood cells)
- Trombocytes (platelets)

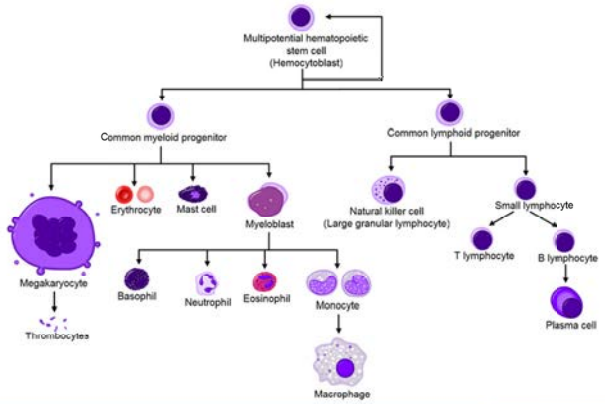


Leucocytes

- Granulocytes
  - Basophils
  - Neutrophils
  - Eozinophils
- Lymphoid cells (agranulocytes)
  - Lymphocytes (B and T)
  - Monocytes

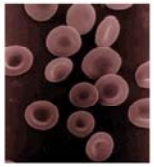


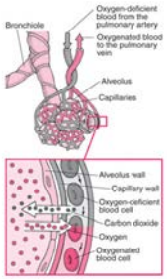
HAEMOPOIESIS / HAEMATOPOESIS



ERYTHROCYTES (RED BLOOD CELLS)

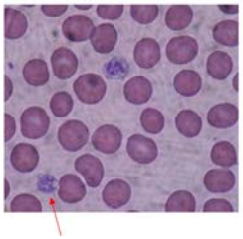
- The most numerous blood cells (about 4-6 millions/mm^3).
- Devoid of a nucleus and have the shape of a biconcave lens (6-8 µm in diameter).
- Rich in hemoglobin
- The mean life spanof erythrocytes is about 120 days
- In the spleen they are phagocyted by macrophages.
- **provide oxygen to tissues** and partly **recover carbon dioxide** produced as waste





THROMBOCYTES (PLATELETS)

- Much smaller than erythrocytes (small sized diskettes, diameter is 2-3 µm).
- Density in the blood is 200 000-300 000 /mm^3.
- Not true cells. They gemmate from big leukocytes called **megakaryocytes.**
- Appear a purple color and are more intense than red cells.
- The main function is **to stop the loss of blood from wounds** (hemostasis).
- The mean life span is 8-12 days.



WHITE BLOOD CELLS

- Leukocytes, or white cells, responsible for **the defense of the organism**.
- Much less numerous than red cells (5 000-7 000 /mm^3).
- Leukocytes divide in two categories: **granulocytes** and **lymphoid cells** (agranulocytes).
- Granules have a different affinity towards neutral, acid or basic stains and give the cytoplasm different colors.

- Granulocytes:
  - Neutrophils 50-70 %
  - Eosinophils 2-4 %
  - Basophils 0,5-1 %
- Lymphoid cells (agranulocytes)
  - Lymphocytes 20-40 %
  - Monocytes 3-8 %

NEUTROPHILS

- The neutrophils are the more common leukocytes.
- They have a diameter of 12-15 µm.
- Nucleus is divided into 2-5 lobes connected by a fine nuclear strand or filament.
- The cytoplasm is transparent because its granules are small and faintly pink colored.
- Very active in phagocytosis of bacteria and are present in large amount in the pus of wounds.
- The mean life span is 6 h–few days.

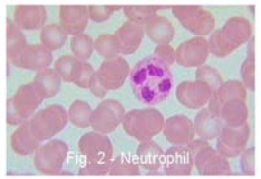
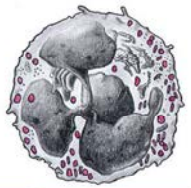


Fig. 2 - Neutrophil



EOSINOPHILS

- Quite rare in the blood.
- They have the same size as the neutrophils (12-15 µm in diameter).
- Generally their nucleus is bi-lobed.
- The cytoplasm is full of granules which assume a characteristic pink-orange color.
- Attack parasites and phagocyte antigen-antibody complexes.
- The mean life span is 8-12 days

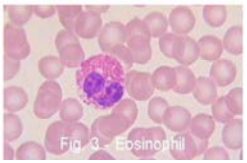
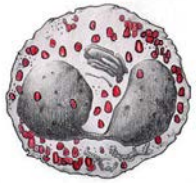


Fig. 3 - Eosinophil



BASOPHILS

- The rarest leukocytes: less than 1 %.
- They are quite small: 9-10 µm in diameter.
- Cytoplasm is very rich in granules which take a dark purple color.
- The nucleus is bi- or tri-lobed, but it is hard to see because of the number of granules which hide it
- Secrete anti-coagulant and vasodilatory substances as histamine and serotonin which mediate the hypersensitivity reaction.

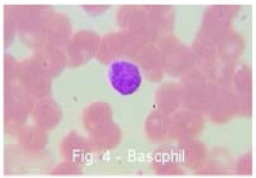
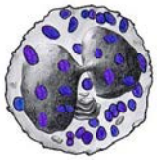


Fig. 4 - Basophil



LYMPHOCYTES

- Little, slightly larger than RBC, cells with a compact round nucleus which occupies nearly all the cellular volume.
- 20-40 % of all leukocytes
- Most lymphocytes circulating in the blood is in a resting state. The lymphocytes of the lymphoid tissues and organs can be activated in a different amount following antigenic stimulation.
- They yield **antibodies** and arrange them on their membrane.
- The main constituents of the immune system which is a defense against the attack of pathogenic micro-organisms such as viruses, bacteria, furgi ...
- The mean life is weeks to years.

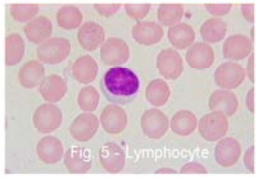


Fig. 5 - Lymphocyte

MONOCYTES

- The biggest leukocytes: 16-20 μm.
- Horseshoe-shaped nucleus, in some cases even bi-lobed. The cytoplasm is transparent, but with an appearance of "ground glass".
- The precursors of **macrophages**. They are larger blood cells, which after attaining maturity in the bone marrow, enter the blood circulation where they stay for 24-36 hours. Then they migrate into the connective tissue, where they become macrophages and move within the tissues.
- In the presence of an inflammation site, monocytes quickly migrate from the blood vessel and start an intense phagocytory activity.
- Also have an intense secretory activity. They produce substances which have defensive functions such as lysozime, interferons and other substances which modulate the functionality of other cells.
- Cooperate in the immune defense; expose molecules of digested bodies on the membrane and present them to more specialized cells, such as B and T lymphocytes.
- The mean life span is from hours to few days.

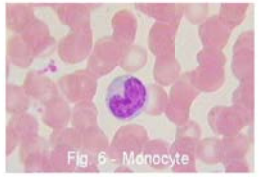
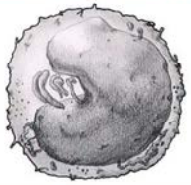


Fig. 6 - Monocyte



IDEAL PROPERTIES OF THE LIGAND

- Specifically labelling following I.V. injection.
- Does not diffuse out of the cell.
- Does not affect cell viability or function.

RADIOLABELLING APPROACHES

Selective approach (*in vivo*)
- specific radioligand (Ab based)
- LeukoScan®: sulesomab, fragment moAb IMMU-MN3 murine Fab'-SH antigranulocyte antibody
- Scintimun®: besilesomab, anti-granulocyte moAb(BW 250/183)

Non-selective approach (*in vitro*)
- Isolation, labelling
- Time consumable
- Appopriate facility, equipement needed

Radionuclide	Half-life	Emission in keV
^{99m}Tc	6 h	140
^{51}Cr	27.7 d	320
^{111}In	67.9 h	171, 245
^{114m}In	49.5 d	190
^{113m}In	100 min	392
^{18}F	110 min	511
Ga-68	68 min	511
Cu-64	12,7 h	511
Zr-89, Sc-44	78,4 h, 3,97 h	511

RADIOLABELLING OF BLOOD CELLS

Clinical application	Blood cellular element	Radionuclide /radiolabel	Radiolabelling approach
Cardiac and vascular imaging	Red cells	^{99m}Tc-pertechnetate	In vivo In vivo/in vitro
Gastrointestinal bleeding	Red cells	^{99m}Tc-pertechnetate	In vivo In vivo/in vitro
Spleen imaging	Denaturated red cells	^{99m}Tc-pertechnetate	In vitro In vivo/in vitro
Blood volume and red cell volume	Red cells	^{99m}Tc-pertechnetate ^{51}Cr-chromate	In vitro
Red cell survival	Red cells	^{51}Cr-chromate	In vitro
Site of red blood cell destruction	Red cells	^{51}Cr-chromate	In vitro
Infection and inflammation	White blood cells	^{111}In-oxine ^{111}In-tropolone ^{99m}Tc-HMPAO	In vitro
Abnormal platelet deposition	Platelets	^{111}In-oxine ^{111}In-tropolone ^{99m}Tc-HMPAO	In vitro

RADIOLABELLING OF BLOOD CELLS

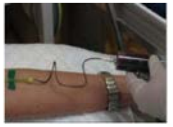
Clinical application	Blood cellular element	Radionuclide /radiolabel	Radiolabelling approach
Quantitative imaging of infection and inflammation	Granulocytes, neutrophils	^{111}In-oxine/tropolone	In vitro
Cell kinetic studies of granulocyte of neutrophils	Granulocytes, neutrophils	^{111}In-oxine/tropolone	In vitro
Lymphocyte migratory patterns and kinetic studies	Lymphocytes	^{111}In-oxine/tropolone	In vitro
Treatment of lymphoid cell malignancies	Lymphocytes	^{111}In-oxine/tropolone	In vitro
Abnormal platelet deposition	Thrombocytes	^{111}In-oxine/tropolone	In vitro
Platelet kinetic and survival studies	Thrombocytes	^{111}In-oxine/tropolone	In vitro
Site of red blood cel destruction	Red cells	^{51}Cr-chromate	In vitro

ISOLATION OF THE CELLS

- Sedimentation
- Centrifugation
- "Density gradient" centrifugation

ISOLATION OF THE WBC

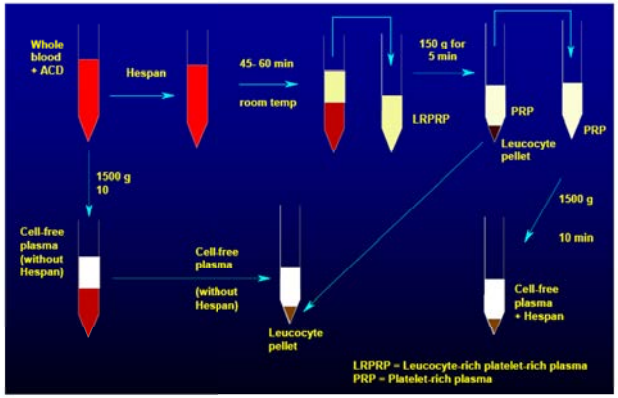
- Erythrocyte sedimentation.
- Plasma removal (leucocyte-rich-platelet-rich).
- Centrifugation at 150g to pellet the leucocytes.
- Platelet-rich plasma centrifugation at 1500 g (cell free plasma).



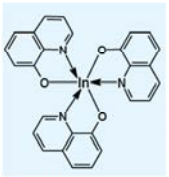
ACD + sedimentation agent

Sedimentation
45 - 60 min RT

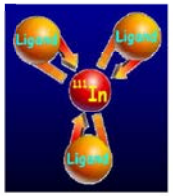
CELL SEPARATION AND RADIOLABELLING



NON-SELECTIVE RADIOLABELLING WITH In-111



^{111}In-oxine



Oxine (8-hydroxyquinoline)

- (binding to transferin in the plasma!)

Tropolone

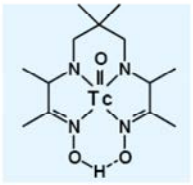
(2-hydroxy-2,4,6 cycloheptatrienone)

- 3:1 complex with indium
- dissociation of the complex in the cell, binding of ^{111}In to intracelular proteins
- Usual injected activity 18.5 MBq

NON-SELECTIVE RADIOLABELLING WITH ^{99m}Tc

HMPAO (*hexamethylpropylene amine oxime), exametazime*

- Meso- and d,l- isomer
- Passive diffusion in the cell
- Conversion in secundary complex (glutation)
- 30 min shelf life
- Stabilisation agents
- Usual injected activity 350 - 500 MBq



^{99m}Tc-HMPAO

51

^{111}In OVER ^{99m}Tc-WBC

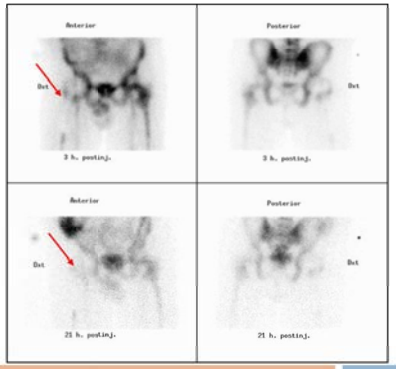
^{111}In-WBC

- Limited availability
- Relative high radiation dose
- Poor image quality
- Good for less acute infection

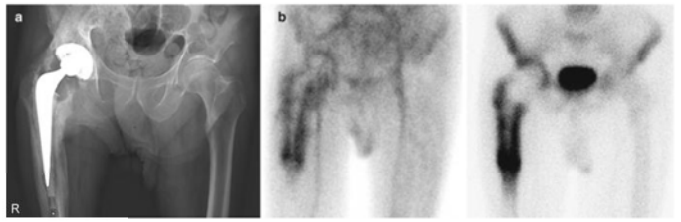
^{99m}Tc-WBC

- Availability and convenience
- Lower radiation dose
- Good only for very acute infection

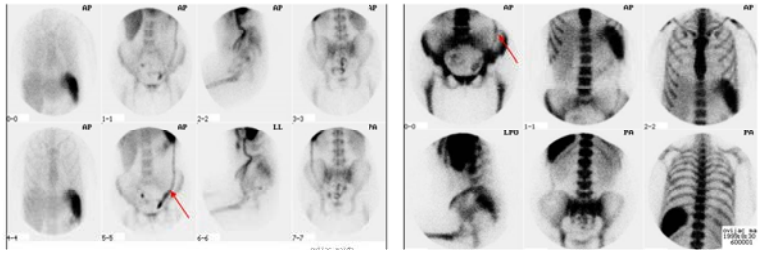
NORMAL SCINTIGRAPHY OF TOTAL HIP PROSTHESIS WITH ^{99m}Tc-HMPAO-WBC



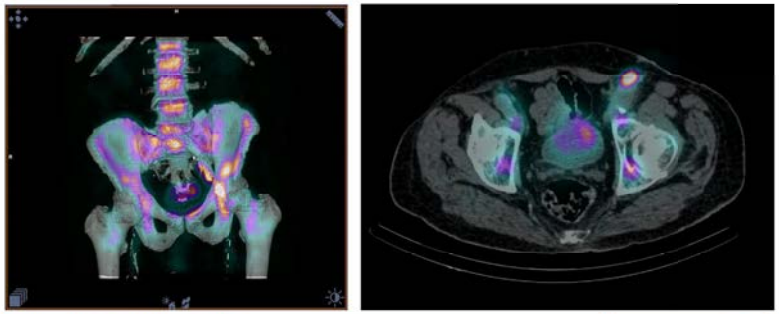
SCINTIGRAPHY OF INFECTED HIP PROSTHESIS WITH ^{99m}Tc-HMPAO-WBC



^{99m}Tc-HMPAO-WBC / ^{99m}Tc-GRANULOSCINT



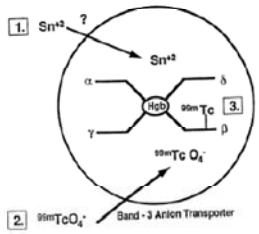
SCINTIGRAPHY OF INFECTED AORTHO-BIFEMORAL GRAFT, LEFT FEMORAL ARTHERY WITH ^{99m}Tc-HMPAO-WBC



SELECTIVE RADIOLABELLING OF RBC WITH ^{99m}Tc

- Pre-tinning
  - Stannous compounds (such as pyrophosphate, MDP) bind to cellular components
- Diffusion of pertechnetate in the cells;
- Reduction of the pertechnetate and binding to the beta chain of haemoglobin
- 10-20 µg/kg BW of Sn(2+)
- Usual injected activity 500 MBq

- *In-vivo*
- *In-vivo / In-vitro*

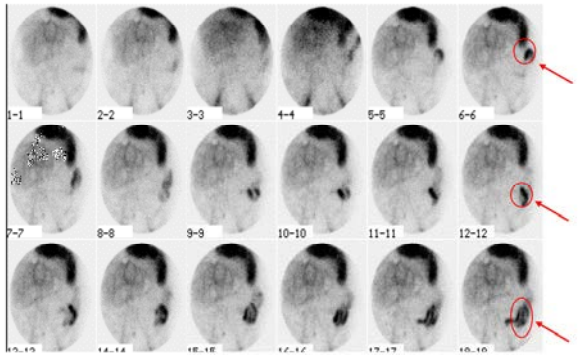


IN-VITRO LABELLING OF RBC WITH ^{99m}Tc

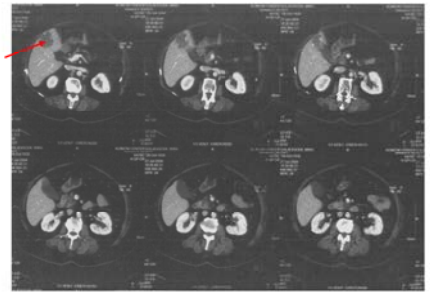
- For determination of red cell and blood volume.
- Patients taking drugs, such as heparin, hydralazine or have previously been given iodinated contrat media.
- The highest labelling efficiency.
- The most stable labelling over time.

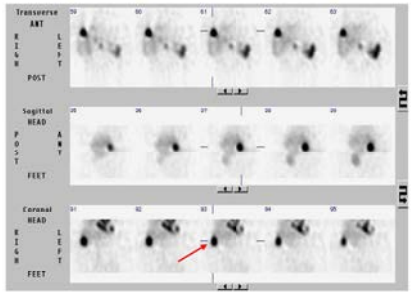
- A small volume of anticoagulated blood (heparin or ACD) is incubated with an aliquot of a reconstituted stannous agent.
- Any excess of Sn^{2+} is oxidized by addition of 0.1% sodium hypochlorite and may be removed by centrifugation.
- Separation of the cells and incubationd with ^{99m}Tc-pertechnetate.
- unbound activity is washed away
- The cells are separated and re-suspended in saline before re-injection.

GI BLEEDING WITH ^{99m}Tc-RBC

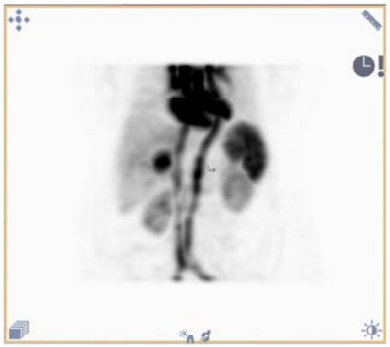


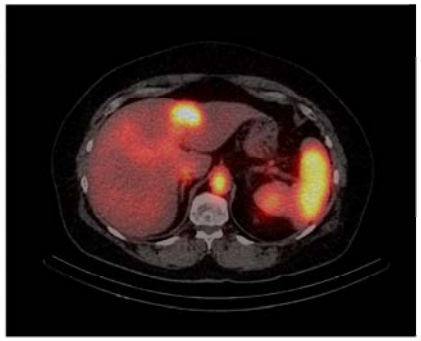
HEMANGIOMA CT / ^{99m}Tc-RBC





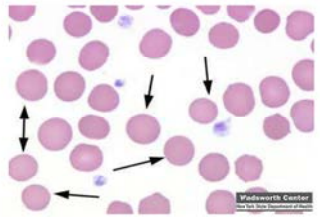
HEMANGIOMA WITH ^{99m}Tc-RBC

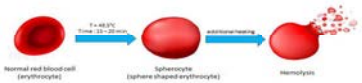




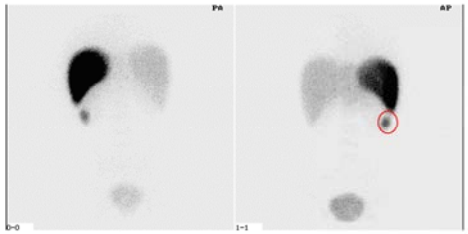
HEAT-DAMAGED ^{99m}Tc-RBC

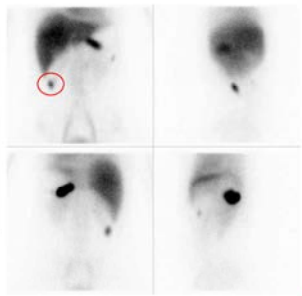
- Spleen imaging.
- Incubation at 49.5°C for approx. 15 minutes
- Fagocytosis of heat damaged ^{99m}Tc-RBC in spleen.





SPLEEN ^{99m}Tc-RBC HEAT DAMAGED





SELECTIVE RADIOLABELLING OF RBC WITH ^{51}Cr

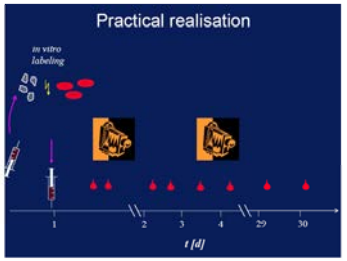
- In-vitro labelling.
- ^{51}Cr sodium chromate to WB containing ACD.
- Incubation 10 -15 min RT.
- Chromate ion freely diffuses into the RBCs.
- Reduction to chromic ion (Cr^{3+}), binding to beta globin chain of the haemoglobin molecule and retaintion in the cell.
- (ascorbic acid to stop the process)
- free chromate is washed away by centrifugation.
- The usual administered activity of ^{51}Cr-RBC for RBC mass and volume determination is 0.8 – 1.5 MBq and 2.0 - 4.0 MBq for survival and sequestration studies.
- 1% washout of the radiolabell daily.

RBC MASS AND VOLUME DETERMINATION

- One part of ^{51}Cr-RBCs is injected to the patient and the other part is used for a standard for further calculations.
- When complete mixing (30 min or longer if the patient has splenomegaly), blood samples are taken for counting in a gamma counter.
- The red cell mass and plasma volume are calculated by using the dilution equations.
- The true blood volume: sum the red cell mass and the plasma volume.
- The total body haematocrit: divide the red cell mass by the true blood volume.
- 1% washout of the radiolabell daily.

RBC SURVIVAL AND SEQUESTRATION STUDIES

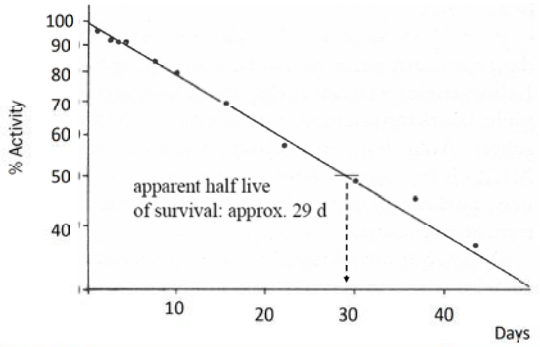
- ^{51}Cr-RBCs are reinjected to the patient
- blood samples are obtained 2-3 times a week for 2 to 4 weeks after injection.



RBC SURVIVAL AND SEQUESTRATION STUDIES

- To eliminate the need for decay correction the samples are counted at the end of the study.
- ^{51}Cr-RBC uptake is monitored using thyroid uptake probe positioning over spleen, liver and heart.
- ^{51}Cr survival curve: % activity of the blood samples over time can be plotted on a semilog paper to linearise the curve. The patient's RBC effective half-life is calculated from the curve (the normal being 25 to 35 days).
- In case of a significant spleenic sequestration of RBCs a significant and progressive increase in spleenic uptake relative to the other expected site of RBCs, like blood pool or liver may be present.

^{51}Cr SURVIVAL CURVE

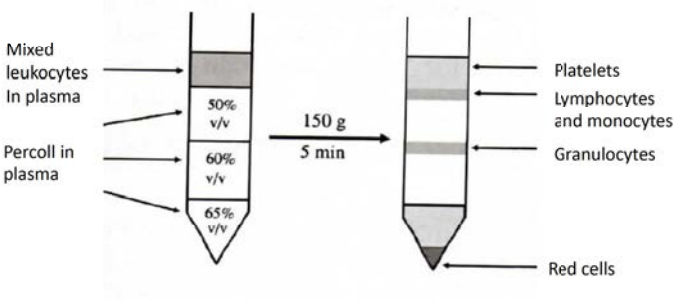


SEPARATION OF NEUTROPHILS AND GRANULOCYTES

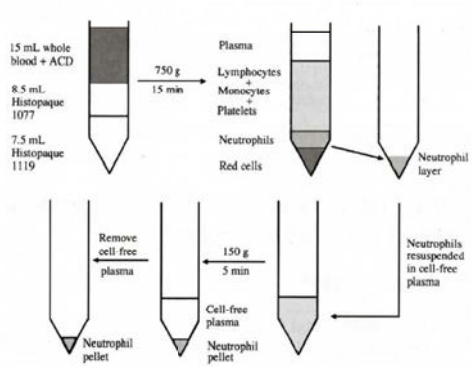
Centrifuging the cells in media with discontinous density gradient

- Percoll-plasma gradients
  (65%, 60% and 50% v/v solutions of Percoll (9 volumes of in 1 volume of 1.5M NaCl) in cell-free plasma
  150 g for 5 min
- Histopaque 119 and Histopaque 1077
  750 g for 15 min

SEPARATION OF GRANULOCYTES



SEPARATION OF NEUTROPHILS



SEPARATION OF LYMPHOCITES

- Lymphoprep; 400 g for 40 min

Lymphoprep™

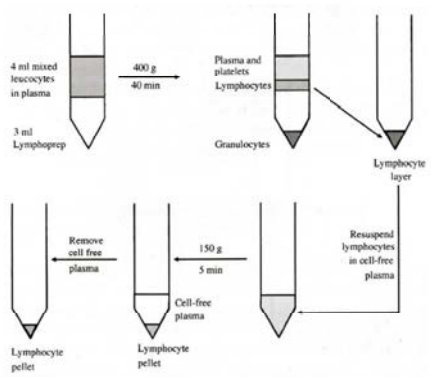
PRODUCT DESCRIPTION
Lymphoprep™ is a ready made, sterile and endotoxin tested solution for the isolation of pure lymphocyte suspensions. The solution contains sodium diatrizoate and polysaccharide in the following concentrations:

Sodium Diatrizoate	9.1% (w/v)
Polysaccharide	5.7% (w/v)

Physical-chemical characteristics:

Density	1.077 ± 0.001 g/ml
Osmolality	290 ± 15 mOsm

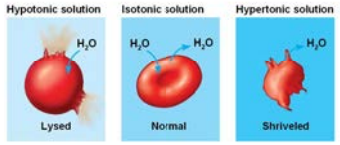
SEPARATION OF LYMPHOCITES



PERC – Module 1, Ljubljana 2025 45

FACTORS AFFECTING RADIOLABELLING

- Plasma concentration and labelling medium
- Types of cells collected
- Method of radiolabelling
- Choice of sedimentation agent
- Choice of anticoagulant
- Presence of disease in patient
- Stability of cell chelator
- Concentration of ligand and radionuclide
- Concentration and number of cells and volume of ingredients
- pH
- Temperature
- Cell damage
- Drugs
- Operator skills



Effect of osmotic pressure on blood cells

PERC – Module 1, Ljubljana 2025 46

QUALITY CONTROL OF RADIOLABELLED CELLS

- Check for clumps.
- Labelling efficiency (the percentage of radioactivity incorporated into the cell)

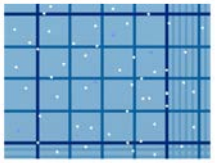
$$\%\ Radiolabelling\ efficiency = \frac{radioactivity\ on\ separated\ cells}{total\ activity\ before\ separation} \times 100\%$$

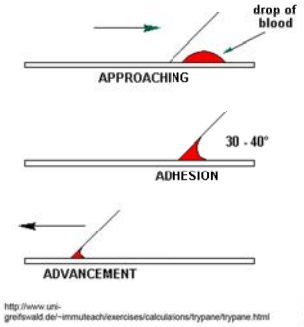
- Cell viability
  - Trypane blue (0.5%)
  - Investigation the biodistribution over a period 15-30 min
- Stability of the radiolabelled cells
  - WBC 2% loss of label per h for ^{111}In and 9% for ^{99m}Tc (always inject ASAP)
  - RBC 1% loss of label per day

PERC – Module 1, Ljubljana 2025 47

TRYPANE BLUE EXCLUSION ASSAY

- Equal volumes (0.1 ml) of cell suspension and 0.5% Trypan Blue (Sigma), mix well, incubate 1-2 min
- Apply on a haemocytometer slide and count.
- Percentage of blue-stained cells represented potential death cells.





PERC – Module 1, Ljubljana 2025 48

PRACTICAL DIFFICULTIES

- Unique specimen.
- Sedimentation step (20 - 60 min)
  - Centrifugation 15 min at 14g (2 - 3 g increments)
- Effects of drugs.
- Innapropriate centrifuge speeds dramatically affect the viability.

DISTRIBUTION CHANGES RESULTING FROM WBC PREPARATION

- False positive: pulmonary Foci (cells not adequalitely resuspended)
- False negative: high background from RBC + platelets
- Low spleen uptake (<20%) more liver and BM: cell fragmentation
- Higher spleen uptake (>50%): cell damage

AFFECT OF DRUGS ON RADIOLABELLING OF WBC

Drug	Affect
Calcium channel blockers (nifedipine)	agranulocytosis
Non-steroidal anti-inflammatory agents	agranulocytosis
Captopril	Bone marrow suppresion
Na valproat	Bone marrow suppresion
Metronidazole	Leukopenia
Allopurinol	Leukopenia
Prednisolone	Adherencde of granulocyte to nylon wool fibres
Alcohol	Adherencde of granulocyte to nylon wool fibres
Aspirin	Adherencde of granulocyte to nylon wool fibres
Lignocaine	Affected uptake of leucocytes into inflammatory areas
Ibuprofen	Affected uptake of leucocytes into inflammatory areas
Adiamycin	Affected uptake of leucocytes into inflammatory areas
Cyclophosphamide	Affected uptake of leucocytes into inflammatory areas
Vincristine	Affected uptake of leucocytes into inflammatory areas
Azathioprine	

AFFECT OF DRUGS ON RADIOLABELLING OF RBC

Drug	Affect
Hydralazine	Oxidation of stannous ions
Methyldopa	Oxidation of stannous ions
Calcium channels blockers	Decrese uptake of stannous ions
Cyclosporine	Interfere with incorporation of stannous ions in RBC
Propranolol	Decreased labelling efficiencies
Chlorotiazide	Decreased labelling efficiencies
Furosemide	Decreased labelling efficiencies
Digoxin	Decreased labelling efficiencies
Prazosin	Decreased labelling efficiencies
Adriamycin	Decreased labelling efficiencies
Sulphasalazine	Decreased labelling efficiencies
Cephalazine	Affect the red cell membrane
Cephalosporins	Affect the red cell membrane
Omeprazole	Affect the red cell membrane
Quinine	Affect the red cell membrane

FACILITIES FOR CELL LABELLING

- Isolator or Biological hazard safety cabinet Grade A in Grade D/B (local regulations!)
- Shielded centrifuge with closed buckets and radionuclide activity calibrator preferably integrated in the grade A environment
- Dedicated room
- Protective (sterile) garment for single labelling
- Regular desinfection of all surfaces: All surfaces should be properly cleaned, decontaminated and disinfected prior to use, after all procedures are completed, and whenever surfaces are overly contaminated
  - 70% isopropanol / denaturated ethanol
  - Chlorhexidine/centrimide mixture (viruses, bacteria, fungi; not spores)
  - Chlorine relising agent or Virkon (after a large blood spill)
  - Klericide B (bacteria, viruses, fungi and spores)

RECOMMENDATIONS

- Be aware of dangers of working with blood samples to personnel.
  - Hepatitis B vaccination (local regulations)
  - Needle stick policy
- Maintain both cell viability and sterility and avoid the operator's exposure to biological and radiation hazard during cell manipulation and radiolabelling.
- Work in aseptic conditions following the EANM guidelines on current Good Radiopharmacy Practice (cGRPP) in the Preparation of Radiopharmaceuticals.
- The use of an open or closed vial system on a laboratory workbench is not an alternative to a controlled sterile environment.
- Cross contamination or mix-up of blood should be prevented at all times.
- Preparation of radiolabelled cells must be performed successive or by different people in different locations.
- Avoid the use of needles.

EANM GUIDELINES

Eur J Nucl Med Mol Imaging (2010) 37:835–841
DOI 10.1007/s00259-010-1393-5

GUIDELINES

Guidelines for the labelling of leucocytes with ^{111}In-oxine

Manel Roca · Erik F. J. de Vries · Francois Jamar · Ora Israel · Alberto Signore

IAEA HUMAN HEALTH SERIES
No. 5
Radiolabelled Autologous Cells: Methods and Standardization for Clinical Use

Eur J Nucl Med Mol Imaging (2010) 37:842–848
DOI 10.1007/s00259-010-1394-4

GUIDELINES

Guidelines for the labelling of leucocytes with ^{99m}Tc-HMPAO

Erik F. J. de Vries · Manel Roca · Francois Jamar · Ora Israel · Alberto Signore

59

REFERENCES

- Ellis BL, Sampson CB. Radiolabelling of blood cells – theory and practice. In: Sampson CB, editor. Textbook of radiopharmacy theory and practice. 3rd ed. Amsterdam: Gordon and Breach Science Publishers; 1999; p. 83-104.
- http://www.funsci.com
- http://en.wikipedia/blood
- http://www.uni-greifswald.de
- Guidelines on current good radiopharmacy practice (cGRPP) in the preparation of radiopharmaceuticals (http://www.eanm.org/scientific_info/guidelines/)
- Solanki KK. Operational guidance on hospital radiopharmacy – A safe and effective approach.IAEA publication in press.
- Guidelines for the safe preparation of radiolabelled blood cells. UK Radiopharmacy Group
- Sampson CB. Complications and difficulties in radiolabelling blood cells: a review. Nucl Med Commun 1996;17:648-58.
- Sampson CB. Adverse reactions and drug interactions with radiopharmaceuticals. Drug Safety 1993;8:280–94.
- Lee HB, Wexler JP, Scharf SC, Blaufox MD. Pharmacologic alterations in Tc-99m binding by red blood cells: concise communication. J Nucl Med 1983;24:397–401.
- Millar AM, Wathen CG, Muir AL. Failure in labelling of red blood cells with ^{99m}Tc: interaction between intravenous cannulae and stannous pyrophosphate. Eur J Nucl Med 1983;8:502-4.
- Karlson KE and Senn LaV Y. Simultaneous Determination of Red Cell Mass and Plasma Volume with Cr^{51} and I^{131} Using a Pulse Height Analyzer.Ann Surg. 1963 August: 158(2): 309–318.

WBC LABELLING PROTOCOL

OVERVIEW

- PATIENT PREPARATION
- PREPARATION OF ANTICOATULANT AND SEDIMENTATION AGENT SOLUTION
- BLOOD SAMPLE EXTRACTION (VENIPUNCURE)
- BLOOD SEDIMENTATION
- LABELLING OF WBCs
- PATIENT INDIVIDUAL DOSE APLICATION

PATIENT AND BLOOD SAMPLE PREPARATION

- Explain the aim of the procedure of examination to the patient.
- Preparation and application of thyroid blocking solution (**3 ml *3% KI*** in **1 dl water, Irenat® (sodium perchlorate)).**

60

PREPARATION OF ANTICOATULANT AND SEDIMENTATION AGENT SOLUTION

antocoagulant :

- **ACD-A** (acid-citrate-dextrose; formulation A)

(European Pharmacopoeia: 0.73 g of anhydrous citric acid, 2.2 g of sodium citrate dihydrate, 2.45 g of dextrose monohydrate in 100 ml of water for injection)

- **heparin**, drawback: microagregation, tendency to adhere to plastic

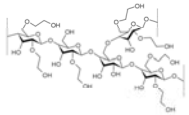
typical concentration ACD-A : blood / 1,5 parts : 8,5 parts

(9ml – 51ml)

PREPARATION OF ANTICOATULANT AND SEDIMENTATION AGENT SOLUTION

sedimentation agent solution

(affecting the charge of the sialic acid groups on the outer membrane of RBCs, faster rouleaux formation):



- **methylcellulose** (2% in saline)
- **6% dextran**, allergy
- **6% HAES** (hespan, hetastarch, 2-hydroxyethyl starch)

sedimentation agent of choice, why? rapid RBC sedimentation, greater leukocyte recovery, no allergy, high molecular weight HAES (200/0,5, 200/0,6, MW aprox. 200kDa)

Voluven® (130/0,4)

- **succilynated gelatin (40 mg/mL),**

Labelling and Clinical Performance of Human Leukocytes Labelled with ^{99m}Tc-HMPAO Using Leukokit® with Gelofusine versus Leukokit® with HES as Sedimentation Agent

typical ratio: hespan : blood / 1 : 10 (3ml:30ml)

can go **up to 1:5** (polycythaemia, sickle-cell anaemia)

BLOOD SAMPLE EXTRACTION (VENIPUNCURE)

- materials needed:

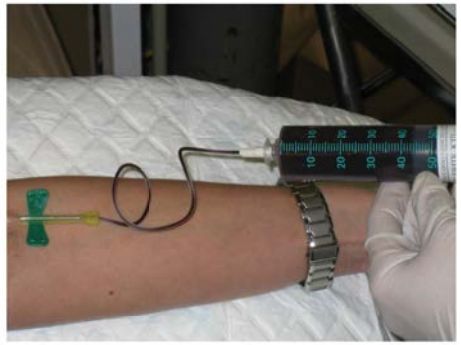
60ml luer lock syringe, 19G needle/buterfly (**≥20G**, prevention of damagirg, high laminar flow- stres)

- **label**: patient name, date of birth, date

- extraction technique:

slow, smooth, mix gently (prevent formation of bubbles, foaming)

BLOOD SAMPLE EXTRACTION(VENIPUNCURE)



movie clip 1

BLOOD SEDIMENTATION

sedimentation (varies)

45 – **60** min (**30** – 45 min)

factors affecting sedimentation:

volume of blood, number of cells, diseases (sickle cell anemia)

no sedimentation?

increase the amount of HAES (1:5)

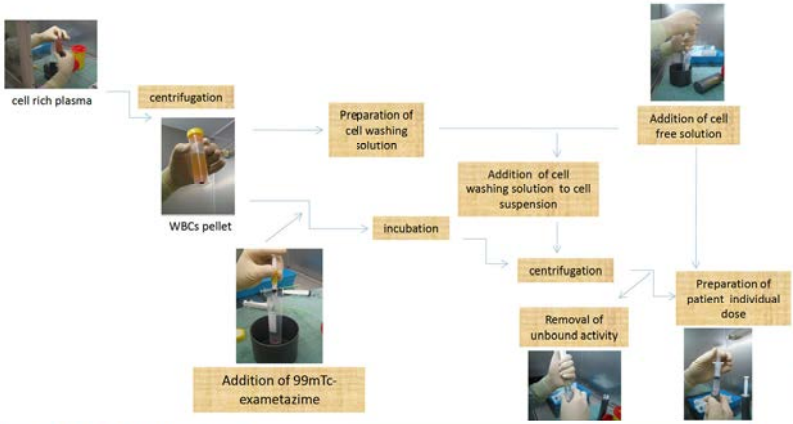
centrifugation, low G (15 min 14G, increase 2-3G, 15min)

BLOOD SEDIMENTATION

BLOOD SEDIMENTATION

	blood	20 min	45 min	60 min	90 min	120 min
WBC (10^9)	4,55	4,77	4,59	3,74	3,54	2,68
RBC (10^{12})	4,95	0,08	0,07	0,06	0,06	0,06
PLT (10^9)	233	192	178	181	177	171

LABELLING OF WBCs



LABELLING OF WBCs

BEFORE PLACING THE MATERIAL IN THE HENCH (AIR LOCK) CHECK INFORMATION ON WORKING SHEET AND LABEL ON SYRINGE FOR ACCORDANCE!!!!!!

BEFORE ENTERANCE OF MATERIAL AND BEGINING OF RADIOLABELING, WHIPE WORKING BENCH IN THE LAF WITH STERILE 70% ETHANOL (IPA) !!!

LABELLING OF WBCs

Separation of LRP (leucocyte rich plasma), from RBCs.

- technique
- composition

	blood	LRP
WBC (10^9)	5,6	2,82
RBC(10^{12})	4,77	0,06
PLT(10^9)	284	195

LABELLING OF WBCs

Video clip 2 (removal of LRP)

LABELLING OF WBCs

Centrifugation of CRP

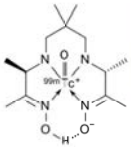
150G / 5 min, no brakes!

our lab: 100G/ 10 min

CELL LABELLING

radiopharmaceutical (Ceretec®)

- ^{99m}Tc-HM-PAO (hexamathylpropyleneamineoxime), exametazime
- ^{99m}Tc in 5+ oxidation state (TcO_4 7+)
- single oxygen atom attached directly to the metal
- metal is coordinated to four nitrogen atoms
- **zero net charge, highly lipophilic**
- two diastereoisomeric forms: meso-, d,1- (superior brain uptake), mixture- cell labeling agent



mechanism:

conversion of the lipophilic ^{99m}Tc-HMPAO complex into a hydrophilic complex by reducing agents such as

glutathione binding of ^{99m}Tc-HMPAO to non diffusible proteins and cell organelles

CELL LABELLING

ADVANTAGE: cells may be labelled in the presence of plasma

DRAWBACK: instability (must be used within 30 min after reconstitution)

- amount of stannous chloride in the kit 7,6 ug (very low concentration of Sn directly related to the degradation from primary to secondary complex)
- age, radioactive concentration of ^{99m}Tc eluate affect the formation of primary complex (extent of degradarion increases: > 2hours old, not eluted in last 24 hours, cause: radiolitic oxydation due to low Sn content)
- methods to enhance the stability and cost effectiveness of 99mTc-HM-PAO

CELL LABELLING

Package insert:

- single dose product

A = 0.37-1.11GBq (10-30 mCi)

V = 5 ml

- for the highest radiochemical purity reconstitute with freshly eluted ^{99m}Tc generator eluate
- use only eluate which was eluted less than 2 hours previously from a generator which was eluted within 24 hours

CELL LABELLING

methods to enhance the stability and cost effectiveness of 99mTc-HM-PAO:

- addition of 0,4 mg of sodium iodide to eluate; (extended shelf life from 2 to 6 hours, oxidising species react with iodide)
- gentinsic acid (effect is pH dependent,practical problems)
- sodium pyrophosphate (weak chelator)
- stabilisation of primary complex: storage in aqeous ethanol, low temperature storage
- reconstitution in saline, subfractionation, low temperature storage (-70,-66, -10)

tin enhacement method:
0,3ml (25ug) HMPAO
0,1ml stannous agent (0,66ug Sn2+)
mix, addition of 400-500MBq
complex stable for 1,5h, up to 15 doses obtained,
CAREFULL VALIDATION, RIGID QC

CELL LABELLING

Prepare 99mTc-HMPAO kit according to the manufacturer's instructions / alternatively:

Labelling of an aliquot of HMPAO (CERETEC®) with $^{99m}TcO_4$

HMPAO (0,3 ml) batch number: date of aliquot preparation:

Sn^{2+} (0,1 ml) batch number: date of aliquot preparation:

- *thaw the aliquots.*
- *transfer alliquot of Sn^{2+} into siringe containing 10 ml of saline; MIX THOROUGHLY and transfer 0,1 ml of diluted Sn^{2+} into 1 ml syringe.*
- *add 0,1 ml of diluted Sn^{2+} into syringe containing aliquot of HMPAO, cap the syringe and MIX THOROUGHLY!*
- *add 600 – 750 MBq $^{99m}TcO_4$ into aliquot of HMPAO, MIX THOROUGHLY and measure the activity.*

eluate: $A_{measured}$ = MBq time:

V = ml V_{total} = ml

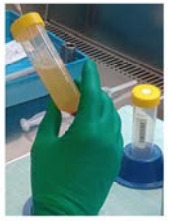
After 2 – 3 minute incubation period add prepared aliquot of ^{99m}Tc-HMPAO to leukocytes in the centrifuge tube

LABELLING OF WBCs

- separation of WBCs pellet from PRP (platelet rich plasma)
- aditional washing step ((CFP, saline,PBS), reduce platelet number)

	LRP	PRP
WBC (10^9)	2,82	0,56
RBC(10^{12})	0,06	0,04
PLT(10^9)	195	189





LABELLING OF WBCs





Video clip 3 (removal of LPP/PRP)

- *addition of prepared aliquot of ^{99m}Tc-HMPAO to leukocytes in the centrifuge tube*

Video clip 4 (addition of RPharm)

LABELLING OF WBCs

- preparation of CFP (cell free plasma), time, G 1500G / 10 min, 2000G / 10 min,
- our lab: 850 G / 10 min

	PRP	CFP (850 G/2000 G)
WBC (10^9)	0,56	0,01 / 0,01
RBC(10^{12})	0,04	0,02 / 0,02
PLT(10^9)	189	87 / 50

LABELLING OF WBCs

- preparation of cell washing solution:

CFP, CFP:saline / 1:3

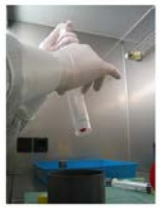
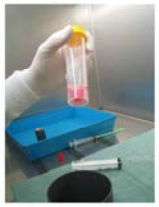
- alternatives: CFP, PBS (pH 7,4), saline
- cell washing (centrifugation,150G/10min....)

Video clip 5 (preparation of cell washing solution)

LABELLING OF WBCs

- resuspension of cell pellet in washing solution (CFP, saline, PBS)



- determination of labelling efficiency

LABELLING OF WBCs

- preparation of patient individual dose (370-740MBq)

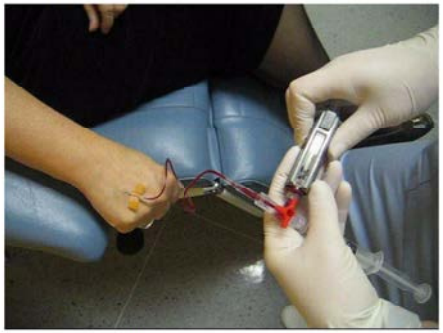
The ^{99m}Tc-HMPAO-labelled WBC should be visually inspected and reinjected into the patient as soon as possible, and not later than 1 h after completion of the labelling procedure




Video clip 6 (preparation of patient individual dose)

INDIVIDUAL DOSE APLICATION

Appropriate technique of application

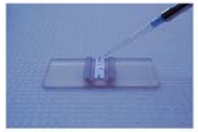
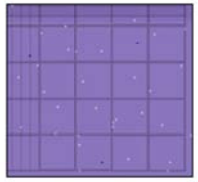


Video clip 7 (aplication of wbc)

QC OF PREPARED RADIOLABELLED CELLS

visual inspection (clumps, clots, fibrin and platelet aggregates)

- should be performed throughout the whole procedure and in particular after resuspending the pellet of cells after centrifugation
- at the end of the procedure and before collecting the labelled cells in the syringe for administration to the patient, the inspection should be performed carefully by gently rotating the vial

labelling efficiency

- LE should be determined by measuring the amount of radioactivity in the supernatant (soluble ^{99m}Tc-compounds) and the pellet (cell-associated ^{99m}Tc) of the labelling solution after centrifugation

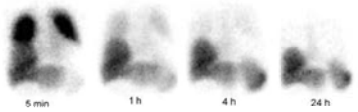
influenced by: volume of Rpharm, temperature,...

- ***viability*** (Trypan blue; 25ul 0,4% Trypan blue /25ul cell suspension)

QC OF PREPARED RADIOLABELLED CELLS

- ***cell subset recovery test*** (ERC/WBC < 3, PLT/WBC <1)
- ***labelling stability***, ^{99m}Tc cell efflux (<10% at 1 hour)

- ***"in vivo" testing*** (early lung uptake, liver-spleen ratio)



- ***Sterility***
- ***LAL***

FACTORS AFFECTING THE LABELLING EFFICIENCY

- plasma concentration (^{111}In-oxine, ^{111}In-tropolone)
- types of cells collected (number; RBC 10^{12}, WBC 10^{9})
- method of radiolabelling
- choice of sedimentation agent
- choice of anticoagulant
- presence od disease in patient
- stabilliy of cell chelator
- concentration of ligand and radionuclide (excess of ligand (3:1))
- concentration and number of cells and volume of ingredients (2× 10^{9} leucocytes are required, neutropenia (<2×10^{3} neutrophils/mm^{3}))
- pH (7,5; 7,2)
- temperature (room T, 37°C)
- cell damage
- drugs
- operator inter-variability

DRUGS AFFECTING THE LABELLING EFFICIENCY

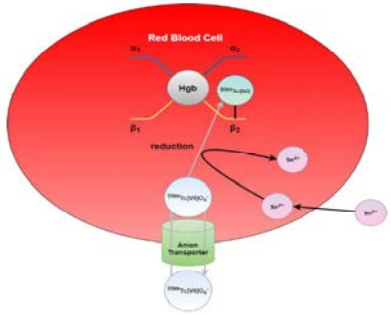
White blood cells		Red blood cells	
calcuim channel blockers(nifedipine)	agranulocytosis	hydralazine	oxidation of stannous ions
captopril	bone marrow suppresion	methyldopa	oxidation of stannous ions
sodium valproate	bone marrow suppresion	calcium channel blockers	Interfering with stannous ion, decreased uptake
metronidazole	leukopenia	cyclosporin	Interfered incorporation into the cell
allopurinol	leukopenia	propranolol	decreased labelling efficiency
prednisolone	adherence to nylon	chlorothiazide	decreased labelling efficiency
alconol	adherence to nylon	furosemide	decreased labelling efficiency
Aspirin	adherence to nylon	digoxin	decreased labelling efficiency
lignocaine	agranulocytosis	prazosin	decreased labelling efficiency
Ibuprofen	agranulocytosis	adriamycin	decreased labelling efficiency
adriamycin	agranulocytosis	sulphasalazine	changes in cell membrane
cyclophosphamide	agranulocytosis	cephalosporins	changes in cell membrane
vincristine	agranulocytosis	omeprazole	changes in cell membrane
azathioprine	Aagranulocytosis	quinine	changes in cell membrane

RBC LABELLING

OVERVIEW

- In-vivo
- In-vivo / In-vitro
- In-vitro

- Pre-tinning
- Stannous compounds (such as pyrophosphate, MDP) bind to cellular components
- Diffusion of pertechnetate in the cells;
- Reduction of the pertechnetate and birding to the beta chain of haemoglobin
- 10-20 µg/kg BW of Sn(2+)
- Usual injected activity 500 MBq



OVERVIEW

- PATIENT PREPARATION
- PREPARATION, APPLICATION OF STANNOUS AGENT SOLUTION (in-vivo, in-vivo/in-vitro)
- PREPARATION OF SYRINGE WITH ANTICOAGULANT
- SAMPLE EXTRACTION(VENIPUNCURE)
- LABELLING OF RBCs
- PATIENT INDIVIDUAL DOSE APLICATION

PATIENT PREPARATION

- explain the aim of the procedure of examination to the patient
- preparation and application of thyroid blocking solution (**3 ml *3% KI*** in **1 dl water, Irenat®**)

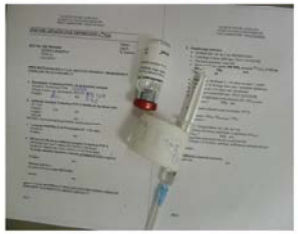
PREPARATION, APPLICATION OF STANNOUS AGENT SOLUTION

- **Dissolution of TecneScan PYP® with 10 ml of saline (acording to spc/package insert)**

batch number TecneScan PYP® solution ®:

batch number saline (BRAUN):

performed: time:



PREPARATION, APPLICATION OF STANNOUS AGENT SOLUTION

Preparation and application of TecneScan PYP® solution (2ml / 70kg body weight)

body weight: *volume:*

performed: time:

applied: time:

optimal amount of stannous ion required for optimal labelling is 10 - 20ug / kg

PREPARATION OF SYRINGE WITH ANTICOAGULANT

materials:

- 6 ml syringe (luer lock)
- drop of Heparin sol. for inj. 25000 i.e./5 ml (neck of syringe), batch number

Attach label with patient's name, date of birth on syringe!!!!

video clip 1

SAMPLE EXTRACTION(VENIPUNCURE)

Extract 4 ml of blood into prepared syringe.
During extraction of sample MIX the content of the syringe GENTLY!

performed: time:

Video Clip 2

LABELLING OF RBCs

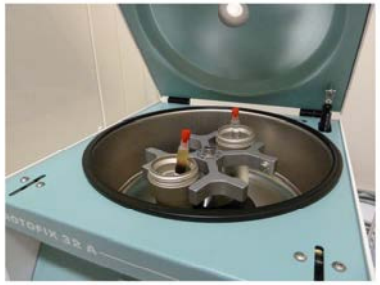
- ***BEFORE PLACING THE MATERIAL IN THE HENCH (AIR LOCK) CHECK INFORMATION ON WORKING SHEET AND LABEL ON SYRINGE FOR ACCORDANCE!!!!!!***
- ***BEFORE ENTERANCE OF MATERIAL AND BEGINING OF RADIOLABELING, WHIPE WORKING BENCH IN THE LAF WITH STERILE 70% ETHANOL (IPA) !!!***

LABELLING OF RBCs

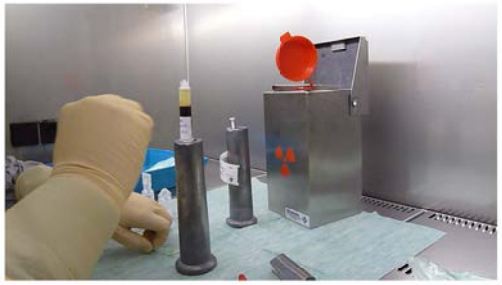
- Addition of saline into the syringe up to total volume 5 ml, MIX GENTLY!
- Centrifugation of the syringe with RBCs for 4 minutes (650 g), NECK OF THE SYRINGE FACING UP!
- Removal of plasma and 1 mm of RBCs.

Additional washing step possible!

LABELLING OF RBCs



4 minutes (650 g)



Video Clip 3

LABELLING OF RBCs

- Resuspend the RBCs in ca 5 ml saline.
- Add 0.3 ml (102 ug Sn) to the RBCs suspension.
- Incubate for 30 minutes at room temperature.
- Remove excess Sn2+ by centrifugation and by resuspension of the cells in 5 ml saline.
- Repeat this wash step.

LABELLING OF RBCs

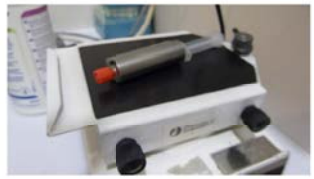
- addition of **500 MBq** of $^{99m}TcO_4$ solution to RBCs (trough the neck of the syringe)
- *smpc: 370-740 MBq (10-20mCi)*

volume of added solution should be as small as possible (V<0.5 ml)

$A_{RBCs\ measured}$ = MBq time:

V_{added} = ml

- mixing the content of syringe thoroughly and incubation for 5 – 10 minutes. (room T, mixing / shaker) *smpc: Incubate 30 minutes at room temperature*



Video Clip 4

LABELLING OF RBCs

- addition of saline into the syringe with radiolabelled cells up to total volume 5 ml, MIX GENTLY!
- centrifugation of the syringe with RBCs for 4 minutes (650 g), NECK OF THE SYRINGE FACING UP!
- removal of supernatant and measurement of it's activity. ($A_{supernatant}$)

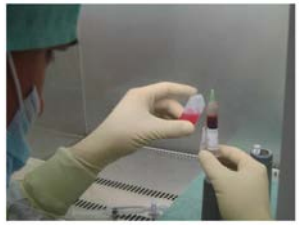
$A_{supernatant}$ = MBq time:

Calculation of labelling efficiency. (expected >93 %)

smpc: labelling yield; should be >85%

Labelling efficiency = $A_{RBCs\ measured} / A_{supernatant} + A_{RBCs\ measured}$

Labelling efficiency = %



Video Clip 5

LABELLING OF RBCs

- addition of saline into the syringe up to total volume 5 ml, MIX GENTLY!
- measurement of the activity of labelled RBCs and adjustment of the dose if necessary. ($A_{patient\ dose}$)

$A_{paticnt\ dosc}$ = MBq

time:

- label syringe containing individual dose.
- (r'pharm, patient's name, date of birth, activity, time, date)

Video Clip 6

THANK YOU FOR YOUR ATTENTION

Microbiology in pharmacy

Assist. Prof. Miša Korva, PhD, assistant at Institute of Microbiology and Immunology, Faculty of Medicine and Research Fellow at Laboratory for diagnostic of zoonoses, Institute of Microbiology and Immunology, has graduated from Faculty of Biotechnology, University of Ljubljana, Slovenia in 2007 and continued her studies in field of Medical Microbiology, under the supervision of prof. dr. Tatjana Avšič Županc. As a young researcher she has joined the Laboratory for diagnostics of zoonoses at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia. She has defended her doctoral thesis, on the pathogenesis of hemorrhagic fever with renal syndrome, in 2011. As a teaching assistant she is involded in the lectures on Emerging pathogens, Biological warfare and Virology at Biotechnical and Medical Faculty. As a post-doc research fellow she has been involved in clinical diagnostics and research of zoonotic vector-borne diseases, mainly viral haemorrhagic fevers and arboviruses. Her research is focused on genetic variability of hantaviruses, TBE, Zika, CCHF and Ebola viruses with the relation to their host-vector-man relationship. She has been working as a coordinator of an intervention response team for diagnostics of highly pathogenic biological angents in the BSL3 laboratory at Institute of Microbiology and Immunology. In 2013 she has joined the European Mobile Laboratory Project (IFS/2011/272-372) and was deployed to Guinea in May 2014 as a part of the international Ebola outbreak response team. She has been involved in several international and national research projects and has published 26 SCI publications.

<u>The lecture has the following learning objectives:</u>

1. Students are able to distinguish biology traits of different microorganisms important for the field of radiopharmacy.
2. Students are able to understand the role of most structural components of microorganisms, important in their identification.
3. Students are able to explain the roles of different new techniques used in microbiology.
4. Students are able to list most important new developments in emerging infections and the role of microbiology in their surveillance.

Summary of the lecture:

Brief overview of microorganisms and their biology

Microbiology is the scientific discipline studying biology of microorganisms. Medical microbiology specifically covers medical aspects of microorganisms (bacteria, viruses, fungi and parasites), infections of humans by these microorganisms, pathogenesis and pathophysiology of these infections and their laboratory diagnostics. One of the most important goals of microbiological laboratory diagnostics is not only determining the causative microorganism (aetiology) of infections but also provide information on susceptibility of the involved microorganisms to antibiotics or other antimicrobial substances.

Biologic basis of microorganisms is different between viruses, bacteria, fungi and parasites so brief introduction covering these differences will be made explained to allow participants to better understand different measures available in the aseptic work and biological safety at work.

Laboratory techniques used in microbiology

Classical techniques in microbiology involve cultivation of microorganisms (i.e. bacteria and fungi) from various materials taken from patient or environment by different techniques and media. If there is a more than one morphological type of bacterial or fungal colony on primary media plate, cultivation of microorganisms is followed by picking the pathogens and make isolation of pure colonies on a new separate plate. After having enough growth of pure colony identification of bacterial colonies is performed either by biochemical methods or by mass spectrometry (MALDI TOFF). Despite relatively high cost of equipment, in recent years the later method has almost entirely replaced the biochemical identification of bacteria in microbiological laboratories and becomes a standard method in identification of bacteria. The method is fast, accurate and less expensive than traditional, biochemical methods. Recent refinements of these methodologies also show promise to provide at least some crude information about the antibiotic susceptibility of some medically important bacteria by detecting specific proteins involved in antibiotic resistance.

In the last twenty years a lot of progress has also been made in other laboratory methods which do not involve cultivation of microorganisms such as molecular methods for specific amplification and detection of nucleic acids from the microorganisms. Direct detection of viruses, parasites, fungi and many bacteria today is predominantly performed by detection of their specific DNA or RNA nucleic acids. By automation, standardization and integration of the processes involved in molecular diagnostics many manufacturers today provide fast, efficient, automated and very sensitive platforms which require less trained personnel and very little hands on time.

Although the price point of many of them is still prohibitive for many users, the indication of future developments is very clear. In the medical microbiological diagnostics the trend would be in direction to highly reliable, highly specific and very rapid (up to 20 minutes) tests which will be available as close to the point of patient care as possible. However, non-cultivation methods should not entirely replace the classical methods, involving cultivation. Without isolates of microorganisms, studies of antimicrobial resistance, epidemiological typing of outbreaks or vaccine predictions, are impossible or are at least technically demanding, expensive or inconclusive.

Disinfection and sterilization

Sterilization means the complete elimination or destruction of all microorganisms including their spores by a chemical or physical means. This is an absolute not a relative term. Sterilization could be achieved by dry or wet (steam) heat. The steam sterilisation is referred as autoclaving and involves treatment of materials with steam heat under sufficient pressure. This is most effective and economical way of sterilization in medical applications as long as materials are thermally stable. Gas or gamma ray sterilization is an option for thermally labile materials such as plastics. Due to importance of the absence of any living microorganism in the materials, sterility control (efficiency of sterilisation process) is mandatory in any sterilization procedures. Chemical, biological indicators are used and physical parameters should be strictly followed and recorded as directed.

Disinfection is a procedure of treatment that eliminates many or all pathogenic microorganisms with the exception of bacterial spores. Reducing or eliminating the microorganisms is possible with heat or chemical agents called disinfectants. Although physical measures such as heat are often more effective, they are often not an option for heat-labile surfaces or objects. Many factors influence the efficiency of disinfectants such are: type and level of contamination, concentration of disinfectant, duration of treatment, humidity, temperature, pH and presence of organic matter. Examples of most resistant microorganisms to disinfection include: bacterial spores (genus *Bacillus*), *Mycobacterium tuberculosis*, hydrophilic viruses without envelope (i.e. rhinoviruses), some fungi (*Cryptococcus* and *Candida*).

Antisepsis is a process involving the destruction or inhibition of microorganisms in or on living tissue thereby limiting or preventing the harmful effects of infection**.**

Sanitization is the process of reducing microbial contamination to an acceptable “safe” level. The process of cleaning objects without necessarily going through sterilization.

Blood borne pathogens as a microbiological risk to personnel in radiopharmacy

Blood borne pathogens are microorganisms that can cause disease in humans through contact with human blood. Most important pathogens from this group include hepatitis B (HBV), hepatitis C (HCV) and human immunodeficiency virus (HIV). In working healthcare environment the most frequent means of acquiring these diseases is through needle sticks or sharps related injuries during medical or laboratory procedures. Many different profiles of workers are at risk for exposure to blood borne pathogens including service personnel such is cleaning and technical staff. Sharps related injuries are common in the healthcare settings and employers must mandatory implement an exposure control plan tailored to the specific worksite with clear details on employee protection measures. These measures encompass personal protection equipment, training, regular medical check-ups, mandatory hepatitis B vaccination and post-exposure evaluation care plan.

Take home messages:

- Microbiology is covering microorganisms with different biological properties important in their function, including bacteria, viruses, fungi and parasites.
- Microbiology plays an important role in different fields of pharmacology such as: production of vaccines, antimicrobial drugs and antiseptics and clean room maintenance.
- Microbiology has undergone extensive change in recent years due to introduction of new sophisticated methods such as MALDI TOFF and massive next generation sequencing (NGS).

References (further reading):

- Angeletti S. Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) in clinical microbiology. J Microbiol Methods. 2017 Jul;138:20–9.
- Patel R, Karon BS. Advances Afoot in Microbiology. Onderdonk AB, editor. J ClinMicrobiol. 2017 Jul;55(7):1984-8.
- Rutala WA, Weber DJ. Disinfection, sterilization, and antisepsis: An overview. Am J Infect Control. 2016 May 2;44(5 Suppl):e1-6.
- Deuffic-Burban S, Delarocque-Astagneau E, Abiteboul D, Bouvet E, Yazdanpanah Y. Blood-borne viruses in health care workers: prevention and management. J ClinVirol. 2011 Sep;52(1):4-10.

MICROBIOLOGY IN PHARMACY

Miša Korva, *Microbiology, PhD Virology, Assistant Professor*
Head of the Laboratory for diagnostics of zoonoses, COVID-19 and BSL-3+

MF UNIVERSITY OF LJUBLJANA Faculty of Medicine
University of Ljubljana | Faculty of Medicine INSTITUTE OF MICROBIOLOGY AND IMMUNOLOGY

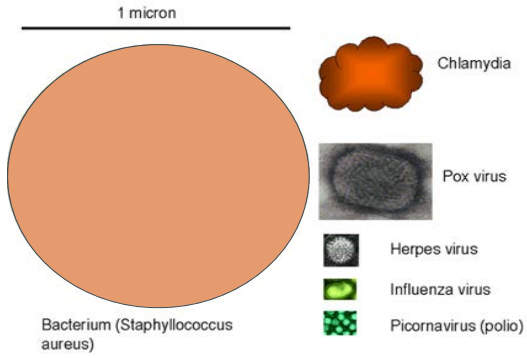
THE MICROBIAL WORLD

- A microbe or microorganism is a member of a large, extremely diverse, group of organisms that are lumped together on the basis that they are TOO SMALL to be seen without microscope
- **Microbes are essential to life** – they are necessary for geochemical cycling, soil fertility, they are used in food production and also as pharmaceutical and industrial compounds…
- **Microbes have a negative side** – infectious diseases, spoilage of food…

GROUPS OF ORGANISMS OF MEDICAL AND PHARMACEUTICAL IMPORTANCE

BACTERIA | VIRUSES
PARASITES | FUNGI

SIZE MATTERS…

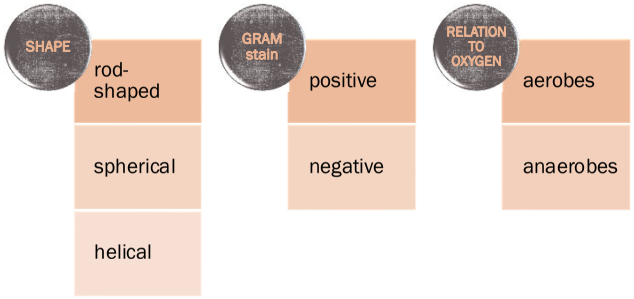


CROSSING POINTS OF MICROBIOLOGY AND PHARMACOLOGY

- Methods for providing microorganisms free environment
- Study of microorganisms and their relations to antimicrobial agents – searching for new antimicrobial drugs
- Studying mechanisms of antimicrobial drug resistance
- Prevention of nosocomial infections

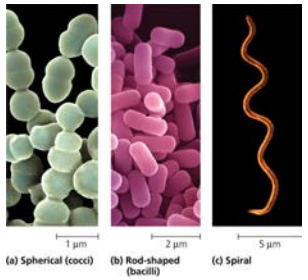
BACTERIA

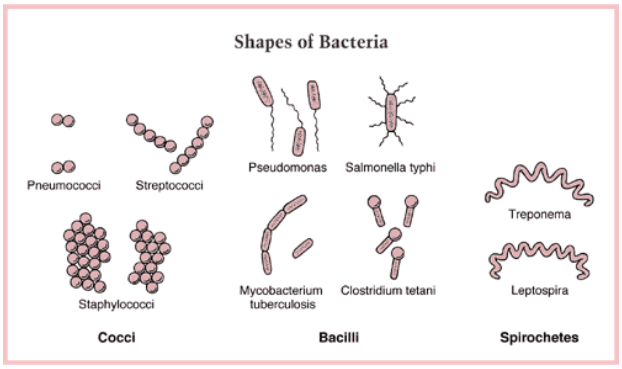
CLASSIFICATION OF BACTERIA IN MEDICAL MICROBIOLOGY



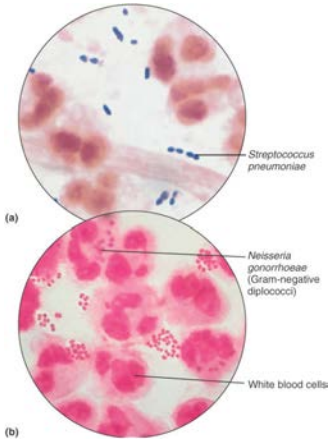
SIZE AND SHAPE OF BACTERIA

- **Size:**
  - Length from 0.3 to 20µm
  - Diameter 1µm
- **Shape:**
  - cocci
  - bacilli (coccobacilli)
  - vibrios
  - spiral bacteria

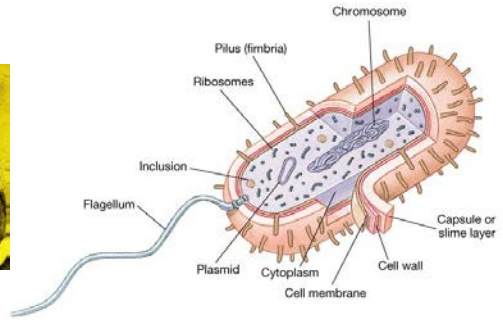




- **Gram positive**
  purple-blue staining
  (crystal violet dye)
- **Gram negative**
  red staining
  (safranin)



STRUCTURE OF BACTERIAL CELL



CYTOPLASM

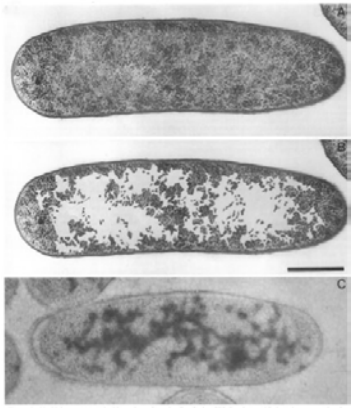
- Semi-liquid state
- Enclosed by cytoplasmic membrane
- 70 % water
- Organic and anorganic substances
- Nucleoid
- Ribosomes
- Plasmids
- Inclusions (starch, iron, lipids, sulphur)

NUCLEOID

- Area of cell with DNA location
- DNA – highly convoluted in protein complexes (deconvoluted length 1.4 mm)
- Usually in the form of **haploid** circular molecule
- At the centre of the cell connected by cell membrane invagination - **mesosome**
- Exemption: *Borrelia burgdorferi* and some Streptomyces species with linear DNA molecule

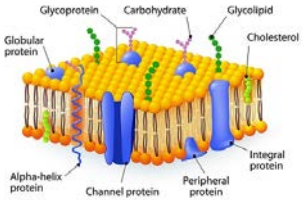
The nucleoid as seen in thin sections of growing *E. coli*

- Panels A and B show the same section;
- In panel B the ribosome-free spaces were enhanced by coloring.
- Panel C shows antibodies which stick to DNA.



CYTOPLASMIC MEMBRANE

Semifluid bilayer of phospholipids with inserted proteins



- **Function of proteins:**
  - Selective permeability and active transport
  - Electron transport and oxidative phosphorylation
  - Transport of hydrolytic enzymes out of the cell
  - Spatial arrangement of multi-enzyme complexes for DNA, cell wall and membrane synthesis
  - Receptors for environmental signals

BACTERIAL CELL WALL

- Rigid framework, shape, osmotic protection
- In cytoplasma osmotic active substances generate pressure up to 5 – 20 atm!!!
- Target of important antibacterial drugs
- Major antigenic determinants
- Endotoxin activity in G- bacteria

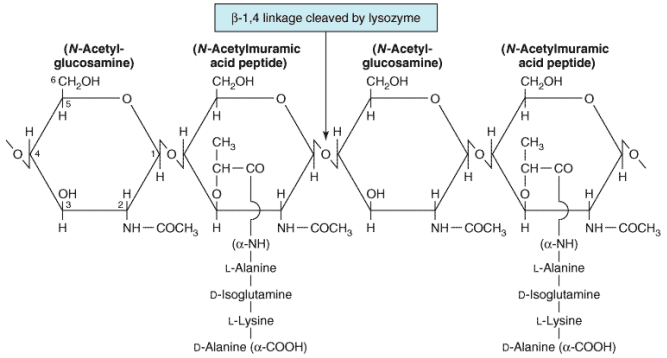
CELL WALL OF G+ BACTERIA

- **Peptidoglycan**
- **Teihoic acids** (up to 50% of cell wall mass), water soluble polymers
  - **Lipoteihoic acid**
    - Connected to glycolipids of cell membrane
  - **Teihoic acid**
    - surrounds peptidoglycan – part of outer layer of cell wall (cell adherence, immune response)

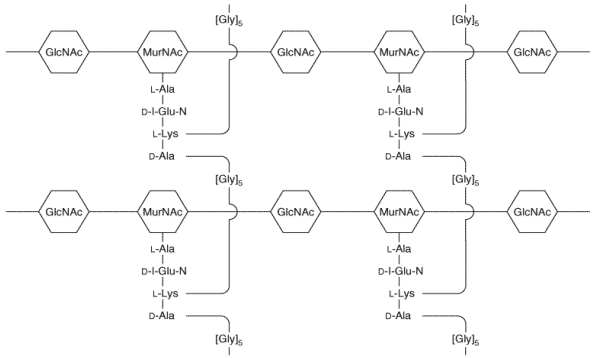
PEPTIDOGLYCAN (MUREIN)

- Complex polymer
- Supports structure of bacterial cell
- Base: long polysaccharide chains
  - alternating molecules of N-acetylglucosamine and N-acetylmuraminic acid
- Difference:
  - G- bacteria 1 or 2 layers
  - G+ bacteria up to 40 layers

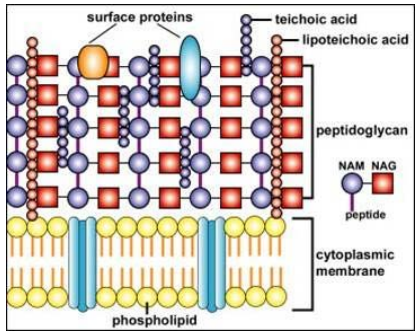
PEPTIDOGLYCAN



Connected by tetrapeptydes in stiff mesh



CELL WALL OF G+ BACTERIA



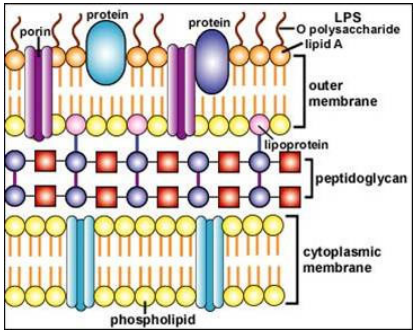
CELL WALL OF G- BACTERIA

- Peptidoglycan (thin; 1-2 layers)

+ 2 additional layers:

- periplasmic space
- outer membrane

CELL WALL OF G- BACTERIA



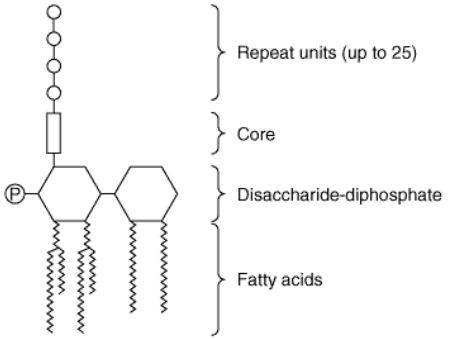
PERIPLASMIC SPACE

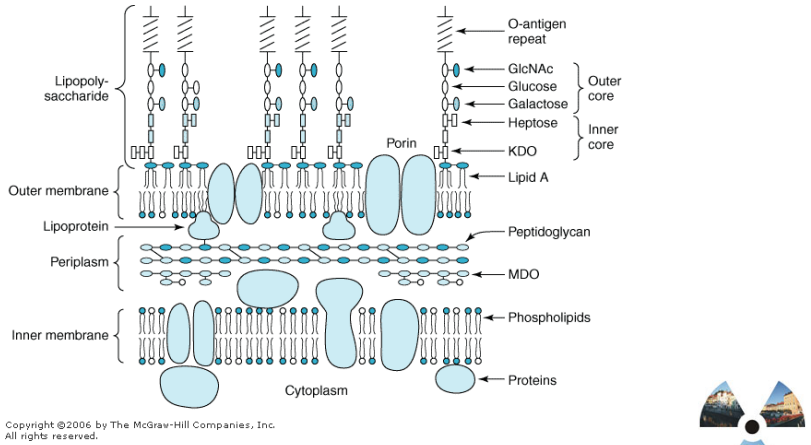
- Stabilized by **lipoprotein** molecules connected to peptidoglycan by protein part, lipid part anchored in outer membrane
- Receptor proteins for diverse molecules
- Hydrolytic enzymes
- Enzymes for antibiotic inactivation: *Beta lactamases, aminoglycoside transferases*

OUTER MEMBRANE

- Asymmetric lipid bilayer with inserted proteins (porines!)
- **Inner layer** similar to phospholipid bilayer of cytoplasmic membrane
- **Outer layer** build from lipopolysaccharide molecules (LPS)

LIPOPOLYSACCHARIDE MOLECULES

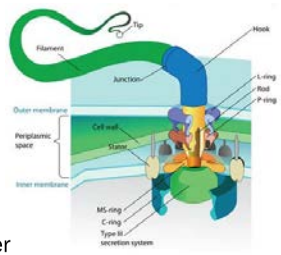




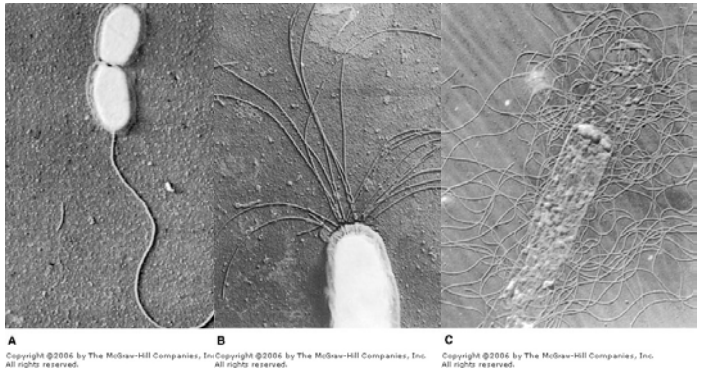
TOXIC EFFECTS OF LPS

- Stimulates macrophages to produce cytokines (interleukin 1, TNF)
- Increase vessels permeability, septic shock
- Toxic effect almost entirely dependent of lipid A
- Some bacteria have rather short and branched terminal polysaccharides –**lipooligosacharides (LOS)**
  - Frequent in mucosal bacteria (ie. *Neisseria)*
- **LOS** could enzymatically bind to human sialic acid and hide from human immune system

FLAGELLA



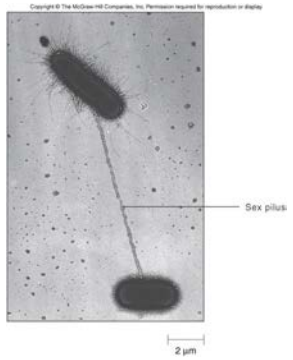
- active movement
- 1000 rpm
- Basic unit protein - **flagellin**
  Units organized in cylindrical shape - fiber
- Anchored in cytoplasmic membrane by basal body
- Uses proton motive force (PMF) to generate of a charge across the membrane
- Target for immune response



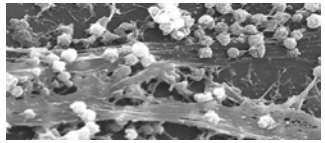
A. *Vibrio metchnikovii* B. *Spirillum serpens* C. *Proteus vulgaris*

PILUS OR FIMBRIAE

- some bacteria have numerous pili
- thinner and shorter than flagella
- anchored in cell wall
- 2 types:
  - **short** – for attachment
  - **long** – sex pilus – exchange of genetic material by conjugation



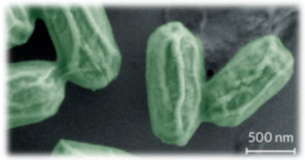
CAPSULE



- Outermost polysaccharide layer densely and compactly surrounds bacterial cell
- Glycocalyx (adherence, biofilm)
- Exemption (!) *B. anthracis* – polypeptide layer

Function: *Evasion from phagocytosis, antibodies and adherence to environmental surfaces*

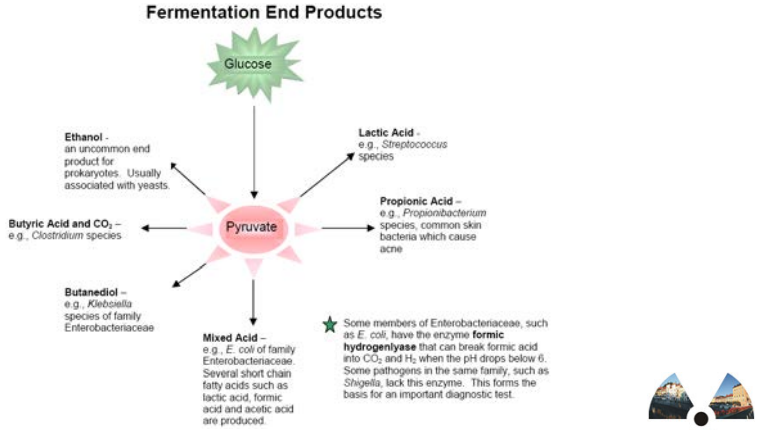
BACTERIAL SPORES



- **Bacteria produce endospores by process called sporulation in metabolically unfavourable conditions** (lack of nutrients, water, heat)
- Metabolically inactive form with complete genome surrounded with very firm, thick wall
- Extremely resistant to drying, heat and chemicals
- Only produced by certain bacterial species (*Clostridium, Bacillus*)

BACTERIAL METABOLISM

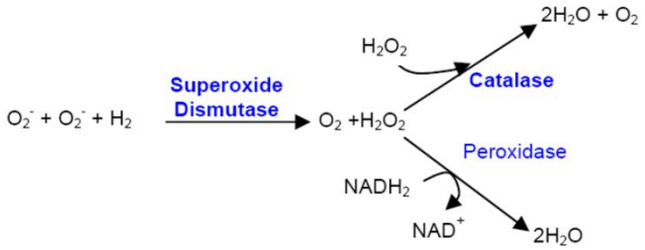
Metabolism similar to the other living organisms



BACTERIAL RELATION TO OXYGEN

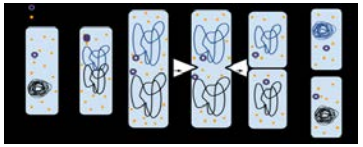
- Strict **aerobes** (no fermentation)
  - *Mycobacterium tuberculosis*
- Strict **anaerobes** (no superoxide dismutase!)
  - *Clostridium*
- **Facultative anaerobes**
  - *Staphylococcus, Streptococcus*
- **Aerotolerant anaerobes**
  - *Lactobacillus*
- **Microaerophilic**
  - *Campylobacter*

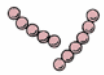
AEROTOLERANCE

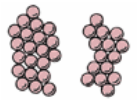


BACTERIAL REPRODUCTION – BINARY FISSION

- Cells remain connected:
  - Chains – streptococci
  - Pairs – pneumococci
  - Grape-like clusters - staphylococci







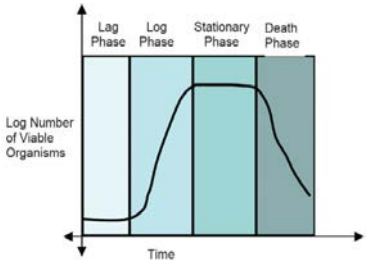
GENERATION TIME

Time needed to produce two daughter cells in optimal conditions

- Most bacteria 15 to 20 min
- 18 do 24 hours – *Mycobacterium tuberculosis*
- Evolutionary advantage
- Calculation: one bacterial cell with gen. time of 20 min produce 10^{21} bacterial cells in 24 hours – 400 tonnes

GROWTH MODEL IN CLOSED SYSTEM

- Components are not replenished and metabolites are not excreted
- Good model for *in-vivo* and *in-vitro* growth of bacteria
- 4 phases



GROWTH FACTORS

NUTRIENTS

PHYSICAL FACTORS

Temperature	Humidity	Atmosphere	pH	Osmotic pressure

- **Psychrophyllic** (<20°C)
- **Mezophyllic** (25 - 40°C)
- **Termophyllic** (> 40°C)

- **Alcalophillic**
- **Neurtophillic**
- **Acidophillic**

BACTERIAL GENETICS

- **Genetic changes:**
  - **Mutations** (base exchanges, insertions, deletions)
  - **Rearrangements of DNA** (inversions, duplications, transpositions)
  - **Accepting foreign DNA from other bacteria** (transduction, conjugation, transformation)
- **Most important for survival:**
  - antibiotic resistance, antigenic changes, pathogenicity islands etc.

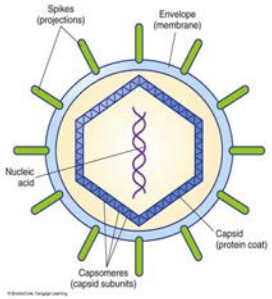
Some viruses are useful:

- Phage typing of bacteria
- Sources of enzymes
- Pesticides
- Anti-bacterial agents
- Anti-cancer agents
- Gene vectors

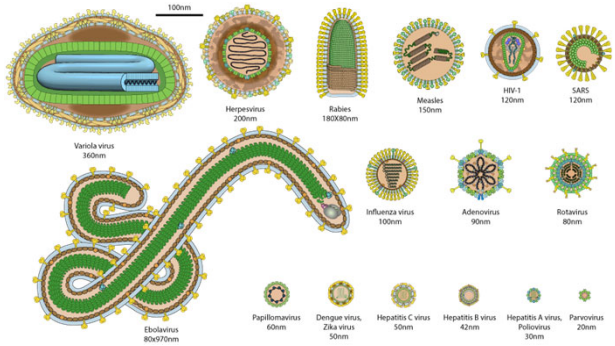
VIRUS STRUCTURE

Viruses are obligate intracellular parasites

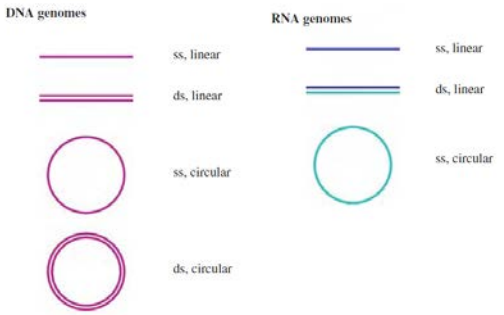
- **Capsid-** surround the center of the virion
- **Nucleocapsid-** combination of the nucleic acid and the capsid
- **Membrane envelope-** surrounds the nucleocapsid
- **Protein spikes-** help viruses attach themselves to the host cell

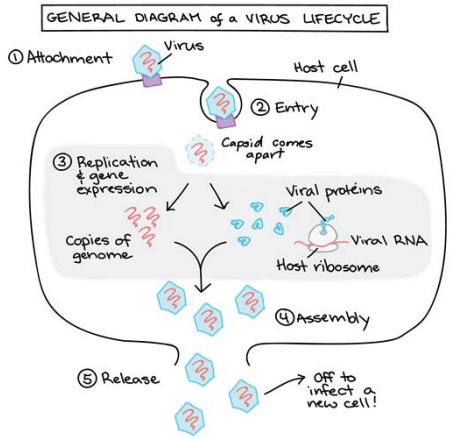


VIRUS SIZE & SHAPE



VIRUS GENOMES

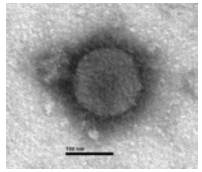


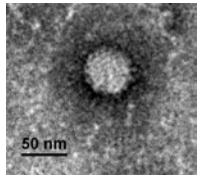


MICROBIAL DIAGNOSTICS

- Direct detection
  - Microscopy (and electron microscopy)
  - Cultivation
  - Detection of antigens
  - Detection of toxins or other products
  - Detection of nucleic acids (PCR)
  - Analysis of protein profiles of intact bacterial cell surface by MALDI-TOF
  - Sequencing (NGS)
- Indirect detection
  - Specific antibodies

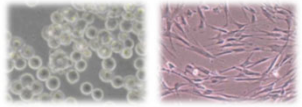
ELECTRON MICROSCOPY





CULTIVATION

- Bacterial cultivation
- Virus cultivation



HIGH CONTAINMENT LABORATORY

Biosafety level 3 **Biosafety level 4**


totally sealed, negative pressure, separate ventilation

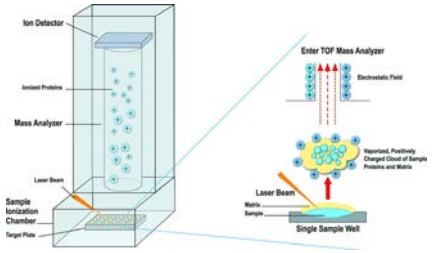
AUTOMATED BACTERIOLOGY LINE

(BD KIESTRA™ LAB)

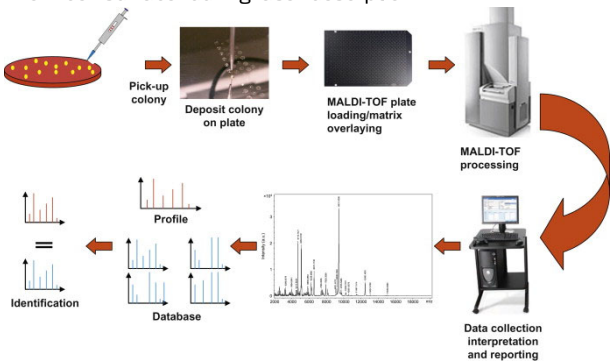


MALDI TOF

- Bacteria identified on the basis of mass/charge ratio
- Soft ionization method
- Ions separated according to their molecular mass and charge

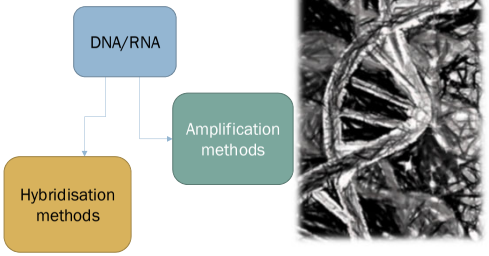


- Reproducible spectrum is reached within minutes
- Each peak corresponds to a molecular fragment released from cell surface during laser desorption

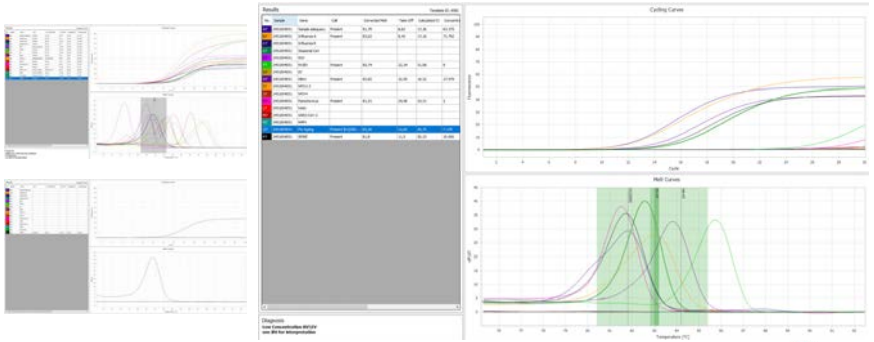


MOLECULAR METHODS

- Techniques/procedures for detecting nucleic acids (DNA/RNA)
- **FEATURES**
  - speed
  - ability to analyze a large number of samples
  - ability to automate
  - high sensitivity
  - regulated specificity
  - special infrastructure/staffing requirements



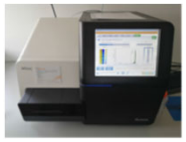
MULTIPLEX PCR (RESPIRATORY PANEL)

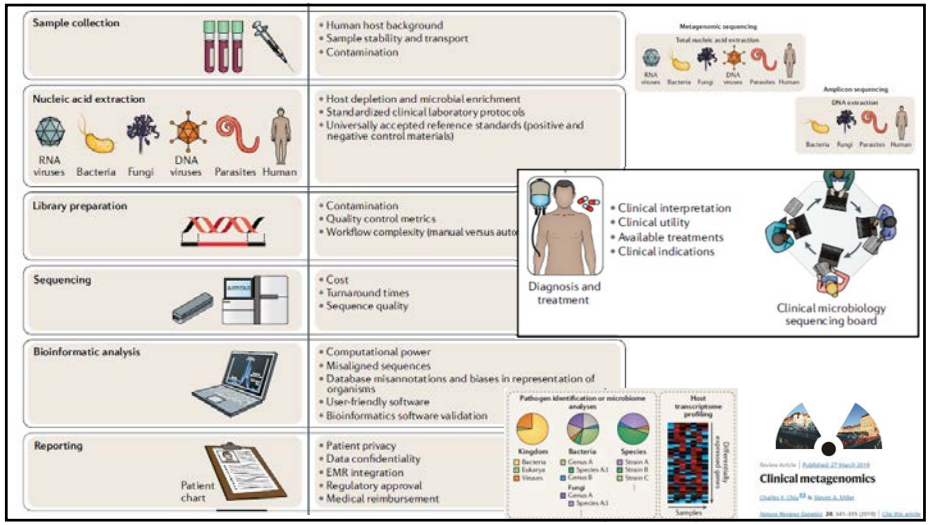


SEQUENCING

Sequencing is the process of determining the nucleic acid sequence.

- The only method that gives us ultimate conformation of the microbial agent
- **Classical sequencing (Sanger)**: Preliminary amplification with PCR / at least partially known target / maximum approx. 1000 bases
- **NGS – short fragments**: target-independent, enormous capacity, reads up to 300 bases, bioinformatically demanding
- **NGS – long fragments**: target-independent, reads of fragments up to 30,000 bases and more: higher error rate





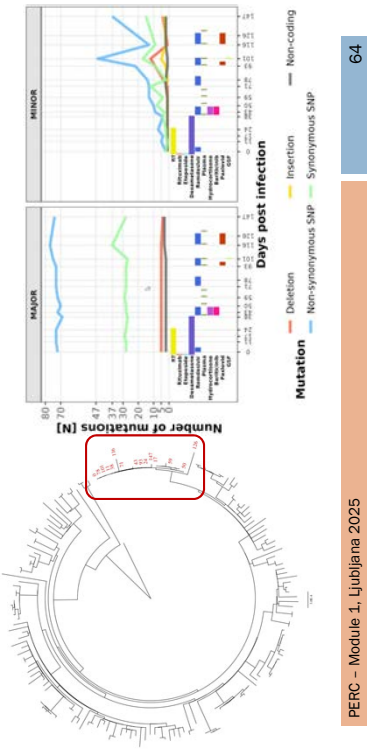
CONTACT TRACING

EBOLA outbreak

Analysis of different genetic virus lineages explained the outbreak

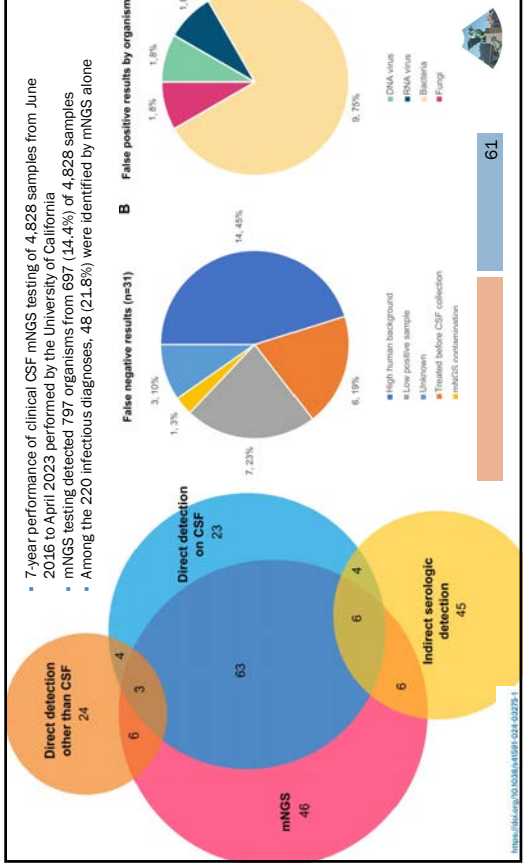
PERC – Module 1, Ljubljana 2025 62

INVESTIGATING VIRUS INTRA-HOST EVOLUTION



PERC – Module 1, Ljubljana 2025 64

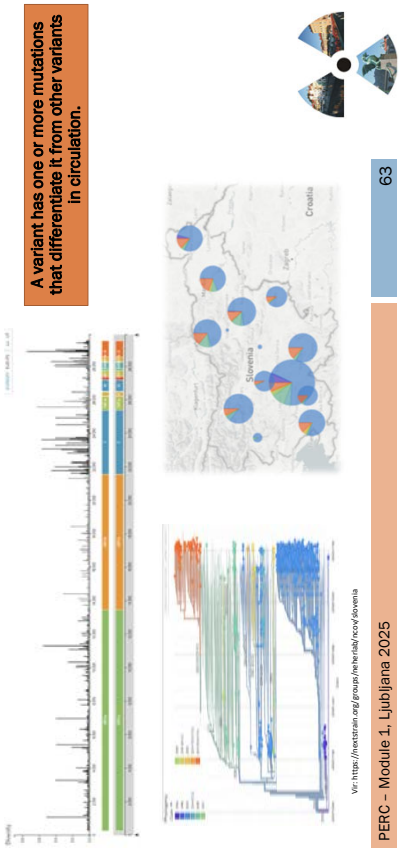
- 7-year performance of clinical CSF mNGS testing of 4,828 samples from June 2016 to April 2023 performed by the University of California
- mNGS testing detected 797 organisms from 697 (14.4%) of 4,828 samples
- Among the 220 infectious diagnoses, 48 (21.8%) were identified by mNGS alone



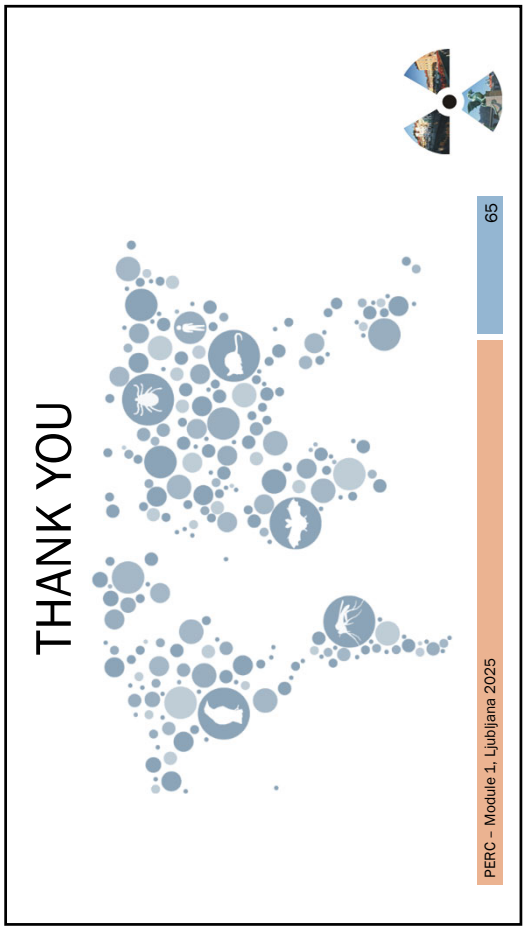
PERC – Module 1, Ljubljana 2025 61

MONITORING OF GENETIC VARIANTS

A variant has one or more mutations that differentiate it from other variants in circulation.



PERC – Module 1, Ljubljana 2025 63


THANK YOU
65
PERC – Module 1, Ljubljana 2025

From gene engineering to modern biologicals

Assoc. Prof. Tomaž Bratkovič, PhD, is a professor of Pharmaceutical Biology and the head of the Department of Pharmaceutical Biology at the Faculty of Pharmacy, University of Ljubljana. He gained his PhD in pharmaceutical sciences in 2007, and was later a short-term research fellow at the Francis Crick Institute, London, UK (January-February 2017) and a visiting professor at the Faculty of Biology, University of Belgrade, Serbia (September-December 2017). He teaches undergraduate courses in Pharmaceutical Biotechnology and Cell Biology. He has supervised 3 PhD students and mentored more than 45 undergraduate students in preparation of diploma or master's theses. His research interests include the study of molecular interactions, both peptide/protein and RNA. His group uses phage-displayed peptide libraries to screen for biologically active ligands; they have been developing peptide enzyme inhibitors and affinity ligands for antibody purification. More recently, he has entered the field of RNA biology, focusing on the functional analysis of small non-coding RNAs, especially small nucleolar RNAs. In the period between 2011 and 2016, he worked as an expert in the biological substances group at the European Pharmacopoeia (European Directorate for the Quality of Medicines, Strasbourg, France). He has authored more than 40 research and review papers.

Learning objectives:

1. To understand the basic principles of recombinant DNA technology.
2. To integrate the understanding of unique properties of biological drugs with specific production processes, analytics, administration route, and storage requirements.
3. To distinguish between innovative biologicals and biosimilars.
4. To understand IgG structure, pharmacokinetics, and pharmacodynamics. To compare the properties of intact antibodies, antibody fragments and synthetic peptides as targeting moieties for radionuclide delivery.
5. To implement the biotechnological knowledge into the field of radiopharmaceuticals.

Lecture summary:

Pharmaceutical biotechnology is a relatively young and fast-growing field in which the principles of biotechnology are applied to the development of drugs. The use of recombinant DNA technology revolutionized the production of modern biological medicines, initially used for protein replacement therapy, and lately allowing for targeted modulation of biochemical pathways and cell functions perturbed in disease. Furthermore, genetic/protein engineering allows for fine-tuning the pharmacokinetics and pharmacodynamics of protein therapeutics, as exemplified by recombinant insulins with different length of action, and fusion proteins, such as

chimeric antibodies in which murine variable fragment involved in antigen binding is grafted onto a human antibody scaffold for enhancing effector functions and decreasing immunogenicity. Biological drugs include, but are not limited to, recombinant enzymes, hormones, cytokines, monoclonal antibodies, synthetic peptides, vaccines and gene therapy products.

Design and development of a biological medicine starts with the selection of optimal expression system that includes an appropriate host organism and vector-harbored gene construct. The vector, such as a plasmid or a modified virus, allows for autonomous replication and transcription of the transferred genetic information. All the therapeutic recombinant protein drugs are produced in a handful of hosts, whose main function is to provide the transcriptional and translational machineries for gene expression. Simple non-glycosylated proteins are typically expressed in the Gram-negative bacterium *Escherichia coli*. On the other hand, complex recombinant therapeutics, especially multidomain proteins that are subject to post-translational modifications (i.e. glycosylation, formation of disulfide bonds, amidation etc.), are produced using eukaryotic organisms or cell lines, such as baker's yeast (*Saccharomyces cerevisiae*), various insect cell lines and Chinese hamster ovary cells. Due to multiple levels of structure, metabolic instability, and complex production processes, protein therapeutics tend to be microheterogeneous, and there is inherent variability between different production batches. This prevents the concept of generic drugs to be applied to biologicals. Instead, copies of biologicals are developed as biosimilar drugs, required to possess essentially the same clinical efficacy and safety profile as reference drugs despite minor structural differences. Most protein drugs are immunogenic, with anti-drug antibodies potentially affecting pharmacokinetics and pharmacodynamics.

Monoclonal antibodies (mAbs) constitute the largest group of recombinant biological therapeutics. mAbs typically display high specificity and high affinity for their targets, and have predictable pharmacokinetics with good safety profiles. These properties, combined with tunable effector functions, make them ideal for addressing various diseases, ranging from cancer, chronic inflammatory and infectious diseases, and many other conditions for which there were few therapeutic options until recently. mAbs also make good delivery agents when conjugated to radionuclides for targeted therapy and as *in vivo* diagnostics. Yet, the large size of intact antibodies limits tissue penetration, and the slow clearance rate leads to substantial radiation delivered to non-target tissues or to low signal-to-noise ratio in imaging applications. Hence, antibody fragments lacking all but the antigen-binding region, synthetic peptides or protein binders (developed on the basis of small stable protein scaffolds using the principles of directed molecular evolution) may represent a viable alternative. Whereas nonmetallic radionuclides (e.g., ^{123}I) can be coupled directly to surface-exposed tyrosine or histidine residues of the protein delivery moiety, radiometals (e.g., ^{68}Ga, ^{89}Zr, or ^{111}In) are typically introduced via bifunctional chelating agents coupled to lysine or cysteine residues. The latter functionalization strategy allows for site-specific conjugation which impacts less on the structural integrity of the protein.

Take-home messages:

- Most modern biologicals are simple or complex proteins, some are synthetic peptides or (viral vector-delivered) nucleic acids.
- Protein production requires endowing an expression host with the genetic information specifying the amino acid sequence of recombinant product, and cultivating the host organism in large fermenters.
- The choice of expression system is primarily dictated by the complexity of the recombinant protein drug. Simple proteins can be efficiently produced in prokaryotic hosts, while complex glycoproteins require the use of eukaryotic hosts.
- Common expression hosts include Gram-negative bacterium *Escherichia coli* and eukaryotic cells, such as yeast (*Saccharomyces cerevisiae* or *Pichia pastoris*) and mammalian cell lines (e.g., Chinese hamster ovary (CHO) cells).
- Structural complexity of proteins requires orthogonal (i.e., complementary) analytical techniques for characterization.
- A protein therapeutic is never considered a single chemical entity, but rather a mixture of similar isoforms.
- Primary protein structure is typically determined by LC-MS/MS, secondary structure can be analyzed by circular dichroism and/or infrared spectroscopy, and tertiary structure is determined using nuclear magnetic resonance or X-ray crystallography. In addition, post-translational modifications, biological activity and impurity profile need to be addressed.
- Protein therapeutics display inherent metabolic, physical and chemical instability. Both, the production process (upstream processing conditions, isolation/purification and formulation) and storage conditions affect protein quality, in turn determining efficacy and safety.
- Due to protein microheterogeneity there are no generic biologic drugs. Rather, the concept of similar biological medicinal products (or biosimilars), which display essentially the same clinical efficacy and safety as the reference biological, was introduced. In EU, all biologicals should be registered through the centralized procedure as either innovative biological medicines or biosimilars.
- All protein therapeutics are potentially immunogenic. Induction of anti-drug antibodies in patients may increase drug clearance or directly negatively affect efficacy via drug neutralization.
- Second generation biopharmaceuticals have tailored pharmacokinetic or pharmacodynamic properties, achieved by either protein engineering (at the level of encoding gene) or metabolic glycoengineering (modulation of glycosyltransferase network in host cells). Some therapeutic proteins are conjugated with polyethylene glycol chain(s) (so-called PEGylation) to make them less immunogenic and to increase their hydrodynamic volume (thus limiting glomerular filtration), and some are acylated to promote association with serum albumin.
- Monoclonal antibodies (mAbs) constitute the largest group of biologicals. mAbs are large multidomain proteins composed of 4 subunits and have complex post-translational modifications.
- The first mAbs were of mouse origin as they were developed via hybridoma technology. Later, chimeric and humanized mAbs, designed using genetic engineering were produced to limit immunogenicity and augment antibody effector functions. Fully human mAbs can be developed via the transgenic mouse platform or phage display technology.
- mAbs have predictable pharmacokinetics and typically display multiple mechanisms of action.

- mAbs make excellent targeting agents for delivery of cytotoxic drugs or radionuclides owing to highly specific and high-affinity interactions with endogenous antigens.
- Antibody fragments, synthetic peptide ligands and specific binders based on small stable protein scaffolds can be used in place of mAbs for targeted delivery. Their smaller size and faster clearance rate are considered an advantage in targeted radiotherapy and radioimaging.
- Conjugation of antibodies with radionuclides is typically indirect (i.e., via bifunctional chelating agents) and can be either random or site-specific.

Suggested further reading:

Crommelin DJA, Sindelar RD, Meibohm B (Eds.) Pharmaceutical biotechnology: Fundamentals and applications. 5th Edition. Springer, Cham, Switzerland, 2019; ISBN 978-3-030-00709-6.
Manis JP. Overview of therapeutic monoclonal antibodies. https://www.uptodate.com/contents/overview-of-therapeutic-monoclonal-antibodies (Accessed: May 5 2023)
Wang L, Wang N, Zhang W, Cheng X, Yan Z, Shao G, Wang X, Wang R, Fu C. Therapeutic peptides: current applications and future directions. *Signal Transduct Target Ther* 2022; 7(1): 48.
Škerlec K, Štrukelj B, Berlec A. Non-immunoglobulin scaffolds: a focus on their targets. *Trends Biotechnol* 2015; 33(7): 408-418.
Lin M, Paolillo V, Le DB, Macapinlac H, Ravizzini GC. Monoclonal antibody based radiopharmaceuticals for imaging and therapy. *Curr Probl Cancer* 2021; 45(5): 100796.
Dewulf J, Adhikari K, Vangestel C, Van Den Wyngaert T, Elvas F. Development of antibody immune-PET/SPECT radiopharmaceuticals for imaging of oncological disorders – an update. *Cancers* 2020; 12(7): 1868.

FROM GENE ENGINEERING TO MODERN BIOLOGICALS

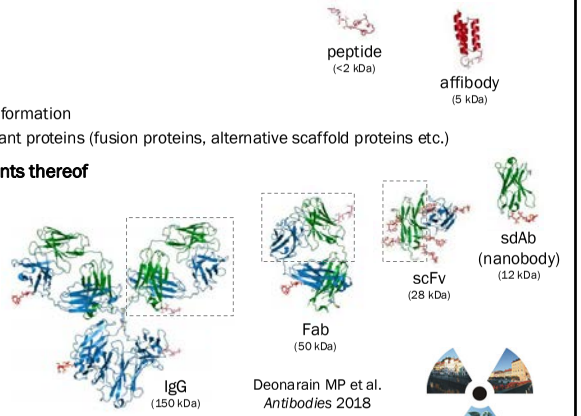
WITH REFERENCE TO RECOMBINANT MONOCLONAL ANTIBODY- AND SYNTHETIC PEPTIDE-BASED RADIOPHARMACEUTICALS

Prof. Tomaž Bratkovič, PhD

UNIVERSITY OF LJUBLJANA | FFA Faculty of Pharmacy

SYNOPSIS

- **recombinant DNA technology**
  - accessing & manipulating genetic information
  - developing and producing recombinant proteins (fusion proteins, alternative scaffold proteins etc.)
- **monoclonal antibodies and fragments thereof**
  - structure, types
  - development
  - PK & PD
- **synthetic peptides**
- **conjugation techniques**

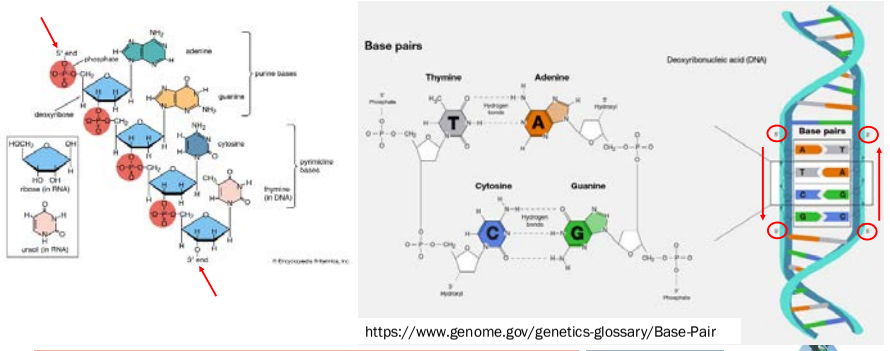


Deonarain MP et al. *Antibodies* 2018

RECOMBINANT DNA TECHNOLOGY;

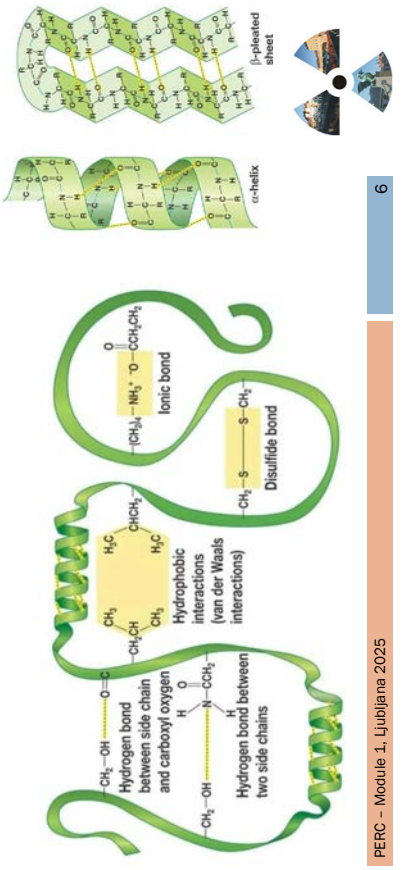
BASIC CONCEPTS & PHARMACEUTICAL BIOTECHNOLOGY

NUCLEIC ACID STRUCTURE

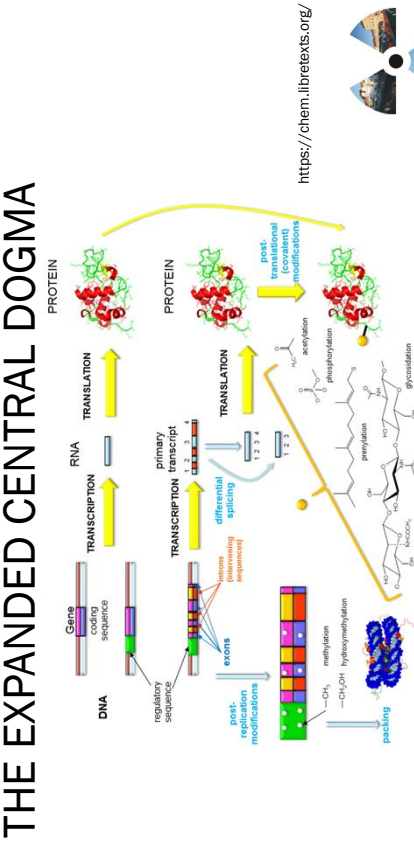


https://www.genome.gov/genetics-glossary/Base-Pair

FORCES STABILIZING PROTEIN STRUCTURE

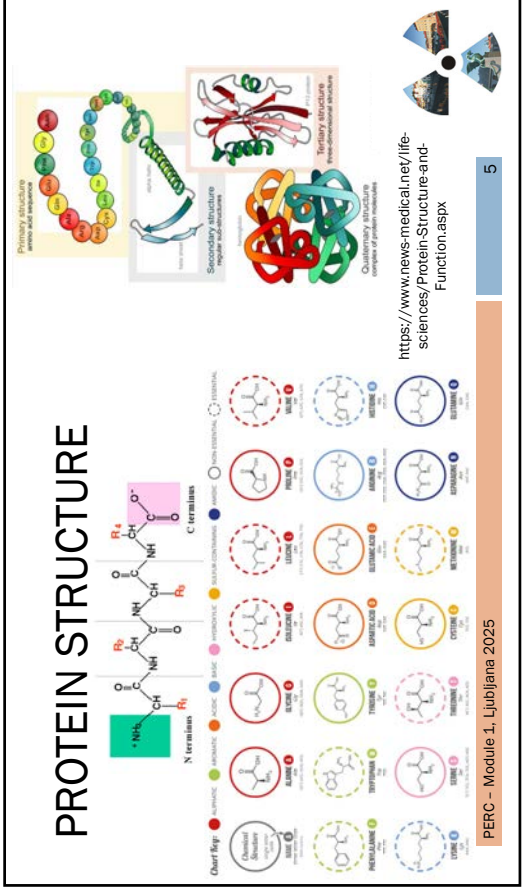


THE EXPANDED CENTRAL DOGMA



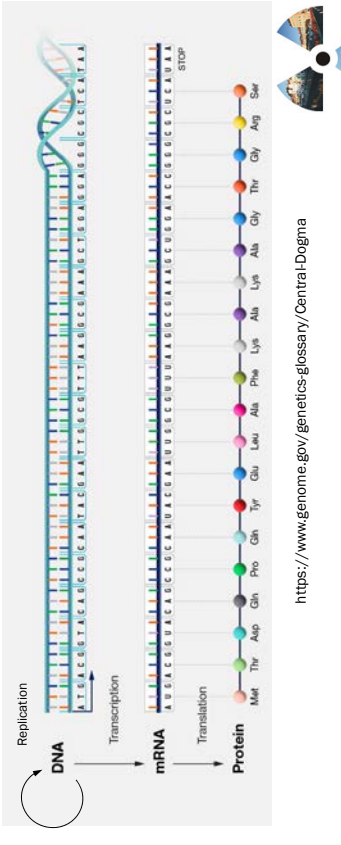
https://chem.libretexts.org/

PROTEIN STRUCTURE



https://www.news-medical.net/life-sciences/Protein-Structure-and-Function.aspx

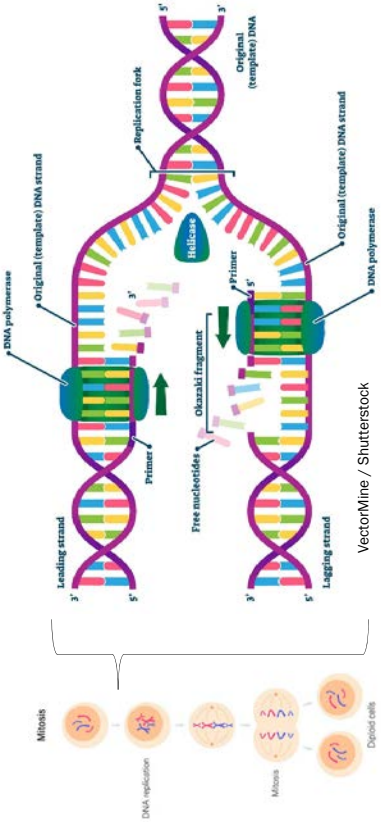
THE CENTRAL DOGMA



https://www.genome.gov/genetics-glossary/Central-Dogma

DNA REPLICATION

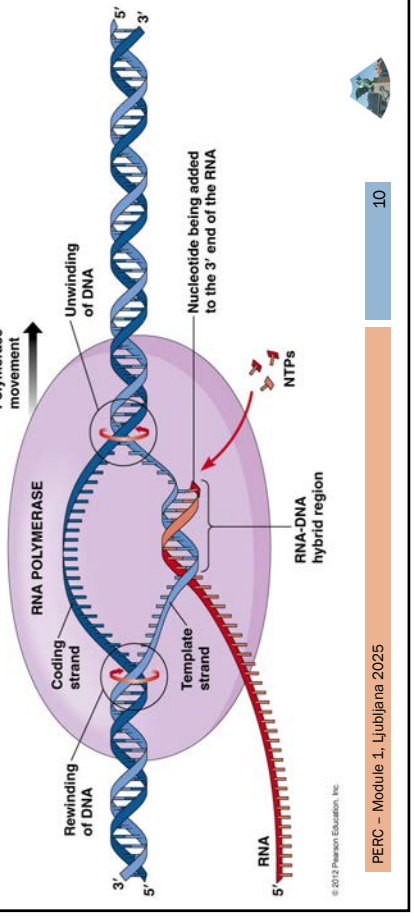
Each DNA strand serves as a template for the synthesis of **complementary strand**. DNA replication is said to be **semiconservative**.



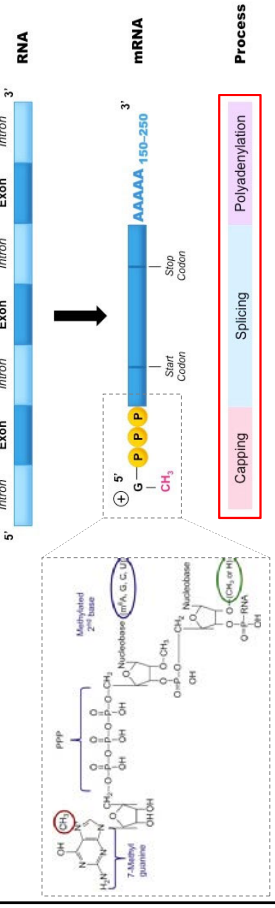
VectorMine / Shutterstock

TRANSCRIPTION

Diverse **transcription factors** direct **RNA polymerases** to gene **promoter** regions and initiate the synthesis of RNA.



POST-TRANSCRIPTIONAL MODIFICATIONS



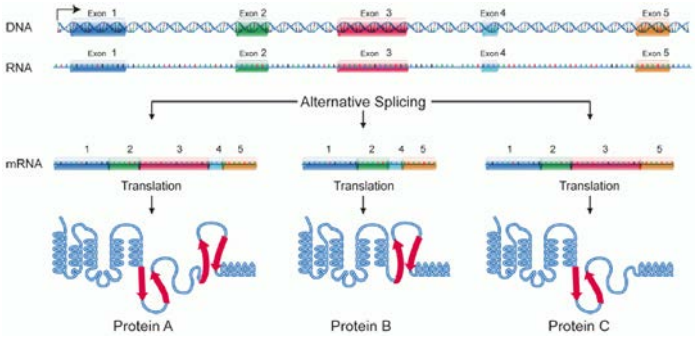
https://ib.bioninja.com.au/higher-level/topic-7-nucleic-acids/72-transcription-and-gene/messenger-rna.html

TRANSCRIPTION

Activator proteins bound to **enhancer sequences** far upstream of promoters can attract transcription factors and RNA polymerase, thereby **augmenting transcription**.

https://www.nature.com/scitable/content/modulation-of-transcription-14711116/

(ALTERNATIVE/DIFFERENTIAL) SPLICING



genomic DNA sequence

primary transcript

mature mRNA isoforms

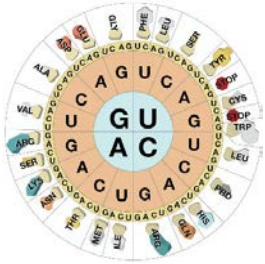
protein isoforms

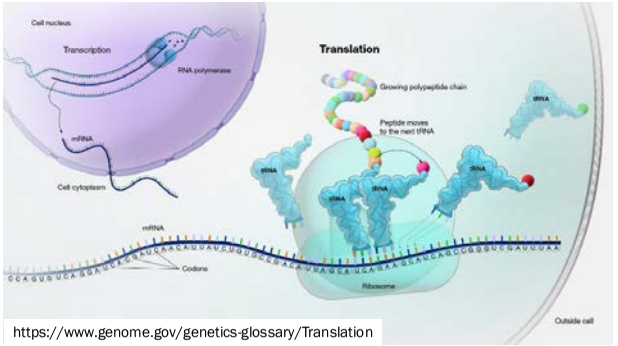
https://en.wikipedia.org/wiki/Alternative_splicing

TRANSLATION

Ribosomes slide along mRNAs and catalyze the polymerization of amino acids (AAs) to form **proteins**. Each AA residue is encoded by a triplet of nucleotides (a **codon**). **Transfer RNAs** (tRNAs) deliver AAs to the ribosome and recognize specific codons with cognate **anticodon loops**.

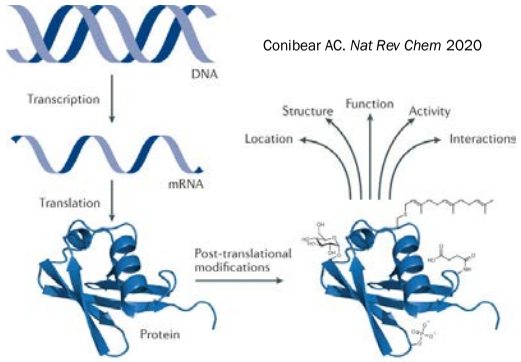
Genetic code





https://www.genome.gov/genetics-glossary/Translation

POST-TRANSLATIONAL MODIFICATIONS



Conibear AC. *Nat Rev Chem* 2020

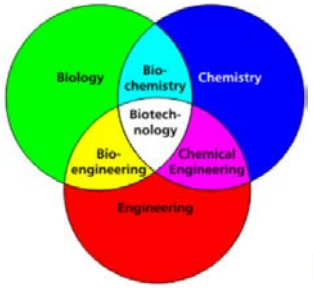
- proteolysis
- glycosylation
- prenylation
- acetylation
- hydroxylation
- phosphorylation
- methylation
- etc.

rDNA TECHNOLOGY

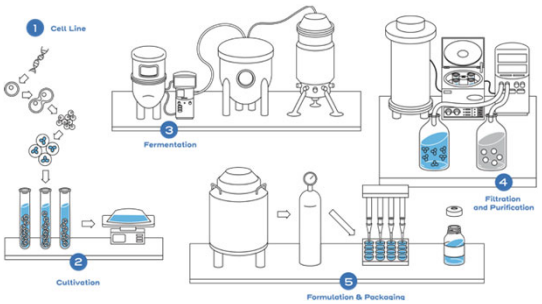
- **genetic engineering**
- **genetic manipulation of DNA** to alter an organism's phenotype
- a group of methods and procedures of **retrieving**, **amplifying**, and **modifying genetic information** and **transfer it from one organism to another**
- creating new genes or new combinations of genes

PHARMACEUTICAL BIOTECHNOLOGY

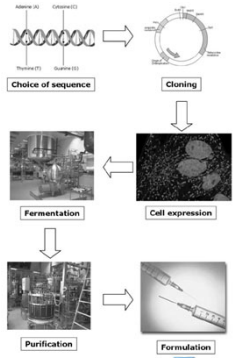
- a scientific discipline in which principles of biotechnology are applied to the development of **biological drugs**
- modern biological drugs are produced via recombinant DNA technology:
  - monoclonal antibodies
  - protein hormones
  - coagulation factors
  - growth factors
  - cytokines
  - vaccines
  - viral vectors for gene therapy
  - etc.



PHARMACEUTICAL BIOTECHNOLOGY



https://www.insidebiosimilars.com/intro/biological-drugs-and-biosimilars/

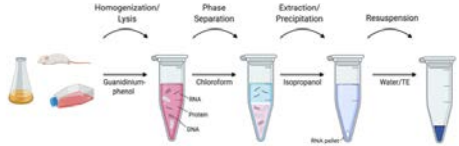


MOLECULAR CLONING

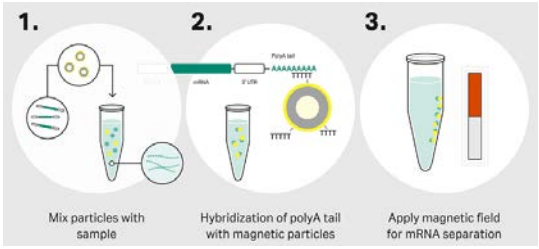
- assembly of recombinant DNA molecules and directing their replication within a host organism
- required tools and materials:
  - enzymes (reverse transcriptase, DNA polymerase, restriction endonucleases, DNA ligase etc.)
  - vectors (e.g. plasmids, viral vectors)
  - genetic material (genomic DNA, mRNA, cDNA, synthetic genes)

mRNA ISOLATION

- **total RNA** extraction using **guanidinium thiocyanate/phenol + chloroform**
- **mRNA** enrichment using **oligo(dT) pull-down**



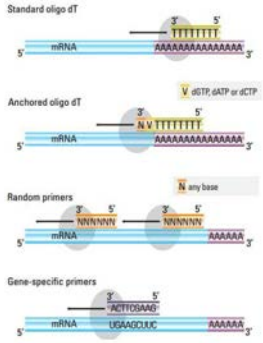
https://www.addgene.org/protocols/kit-free-rna-extraction/



https://www.cytivalifesciences.com/en/us/news-center/mrna-purification-after-mrna-synthesis-10001

REVERSE TRANSCRIPTION

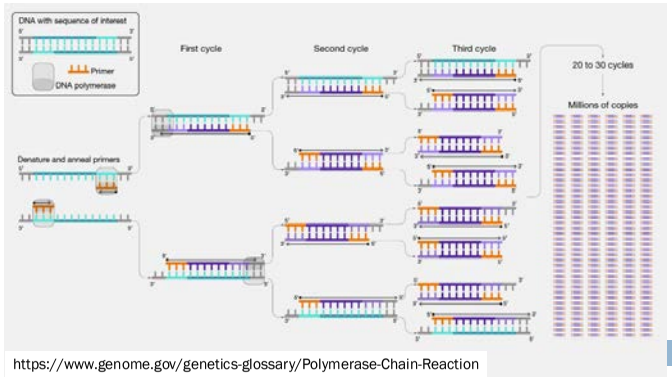
- synthesis of **complementary DNA** (**cDNA**) based on (m)RNA sequence (intron-free sequence!)
- cDNA stable and can be amplified using **polymerase chain reaction** (**PCR**)



ThermoFisher Scientific

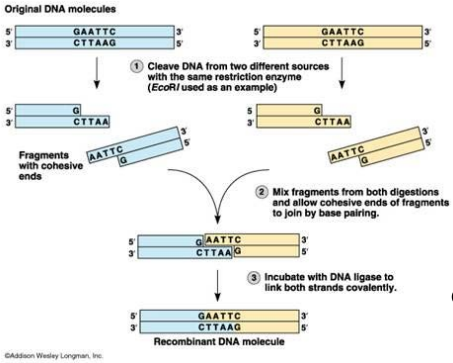
POLYMERASE CHAIN REACTION

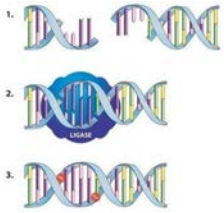
Exponential copying of specific DNA sequence *in vitro*.



https://www.genome.gov/genetics-glossary/Polymerase-Chain-Reaction

RESTRICTION DIGESTION & LIGATION



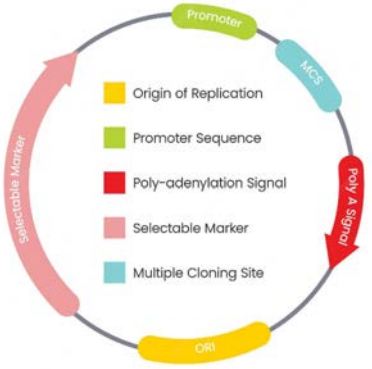


https://www.sciencelearn.org.nz/resources/2034-dna-ligation

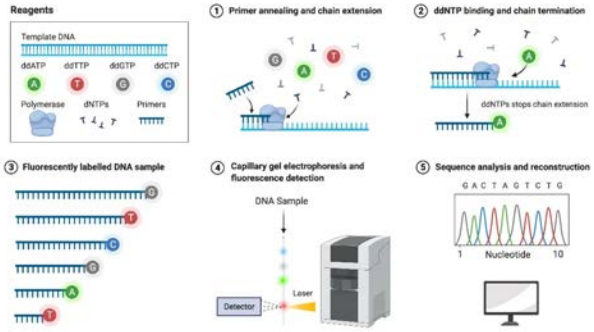
Other methods of recombining DNA molecules *in vitro* and *in vivo* available!

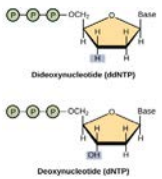
VECTORS

- **vehicles** for carrying foreign nucleic sequence in a recipient cell
- supporting the **copying of genetic information**
- supporting **expression of genetic information** (transcription)
- harbor selectable markers conferring traits suitable for artificial selection



DNA SEQUENCING





https://microbeonline.com/dna-sequencing-sanger-sequencing-method/

BIOLOGICAL DATABASES

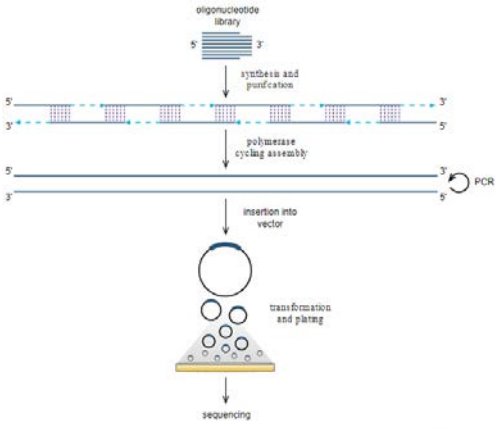
- genome & transcripts (e.g. EMBL, GenBank)
- proteins (e.g. UniProtKB, PDB), etc.
- freely accessible on line
- data available for many species

BIOINFORMATIC TOOLS

- codon optimization
- restriction digestion
- primer design
- folding/3D structure prediction
- etc.

GENE SYNTHESIS

- assembly from short synthetic oligonucleotides
- oligos are routinely produced via solid-phase synthesis
- large genes or even entire plasmids can be constructed

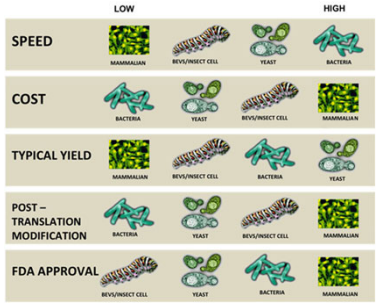


EXPRESSION SYSTEMS

- **gene + vector + host organism**
- the host provides transcription and translation mechanisms for gene expression
- different host organisms are used for production of therapeutic proteins:
  - **bacteria (*E. coli*)**
  - **yeast (*S. cerevisiae*, *P. pastoris*)**
  - **insect cells**
  - **mammalian cells**
  - plant cells
  - transgenic animals (goats, rabbits)

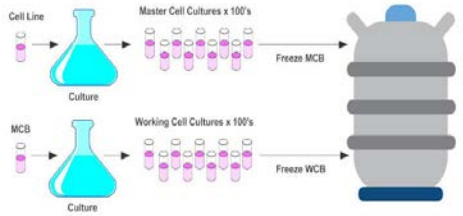
EXPRESSION SYSTEMS

- each system has its own advantages and disadvantages
- choice is driven by the **nature of recombinant protein** and **economic considerations**



UPSTREAM PROCESSING

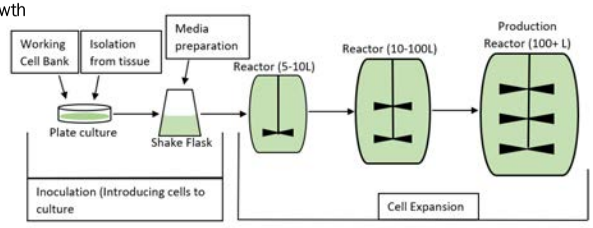
- a number of cell clones are screened for optimal growth and production capacity/quality
- the chosen clone is expanded and **master cell bank (MCB)** is prepared (stored frozen)
- **working cell bank (WCB)** is prepared by expanding MCB



https://mastercellbank.com/bio-technology/master-cell-banking

UPSTREAM PROCESSING

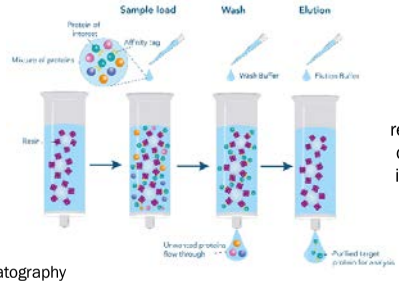
- WCB is used to inoculate growth medium for **cell expansion**



https://bioprocessingexplained.home.blog/2019/09/01/what-is-upstream-processing

DOWNSTREAM PROCESSING

- **cell separation**
  - filtration
  - centrifugation
- **concentration & capture**
  - ultrafiltration/diafiltration
  - affinity chromatography
- **intermediate purification**
  - ion-exchange chromatography
  - rpHPLC
- **polishing**
  - hydrophobic interaction chromatography
  - size exclusion chromatography
- **viral clearance**

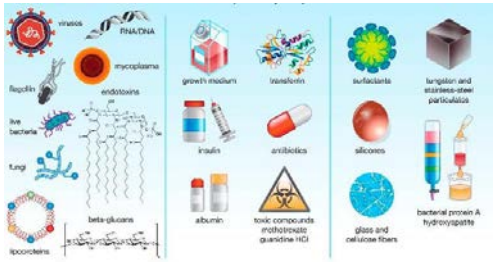


https://www.iba-lifesciences.com/applications/protein-affinity-chromatography/

Diverse **separation methods** (exploiting differences in physicochemical properties among recombinant product and contaminants) are used in succession to remove most impurities.

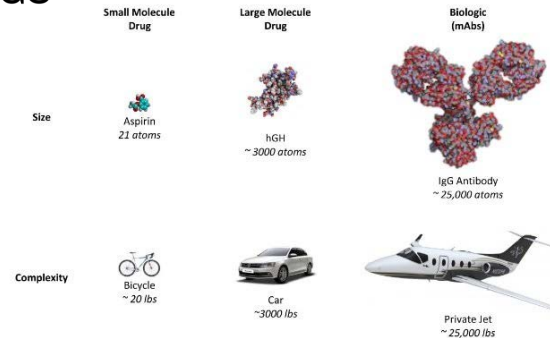
IMPURITIES IN RECOMBINANT BIOTHERAPEUTICS

- **host-related**
  - endotoxins (LPS - *E. coli*)
  - glycosylation variants
  - host cell proteins and nucleic acids
  - adventitious agents (viruses)
- **process-related**
  - growth medium components
  - chromatography column materials
  - purification reagents
- **product-related**
  - denatured proteins → aggregates
  - conformational isomers
  - oxidized species
  - deamidated species
  - disulfide pairing variants
  - protein fragments



Holley CK & Dobrovolskaia MA. *Molecules* 2021

UNIQUE PROPERTIES OF BIOLOGICAL DRUGS

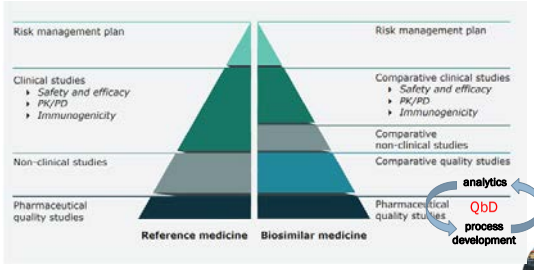


UNIQUE PROPERTIES OF BIOLOGICAL DRUGS

- numerous **isoforms** (similar chemical entities)
- inherent minor structural/functional variability (**microheterogeneity**) within and slightly different impurity profiles between different batches
- sometimes multiple mechanisms of action
- generics NOT possible
- **similar biological medicine** (**biosimilar**):
  - *biological medicine highly similar to another biological medicine already approved in the EU (i.e. reference medicine);*
  - essentially the same efficacy and safety profile, minor structural differences recognized

BIOSIMILARS

- all studies are of **comparative** nature (comparison to a biological medicine already authorized in the EU = **reference** drug)
- production process is fine-tuned for the biosimilar to match the reference product as closely as possible



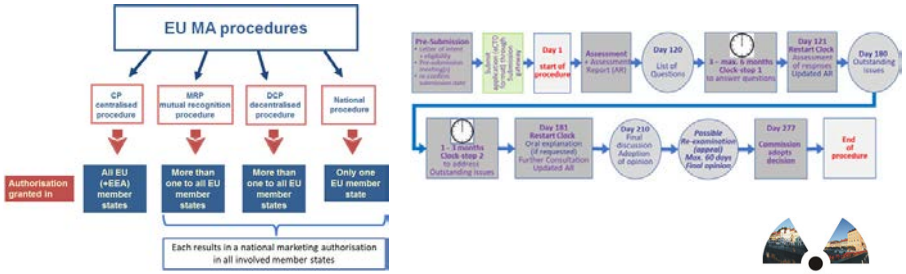
https://www.ema.europa.eu/en/human-regulatory/overview/biosimilar-medicines-overview

RECOMBINANT PRODUCT CHARACTERIZATION

- due to many layers of protein structure **orthogonal methods** providing complementary information are required
- insight into:
  - AA composition
  - AA sequence
  - secondary structure
  - 3D fold
  - molecular weight
  - isoelectric point
  - hydrophilicity/hydrophobicity
  - hydrodynamic volume
  - specific activity
  - position of disulfide bonds
  - glycan structure
  - host cell proteins
  - residual DNA
  - endotoxins
  - sterility
  - etc.

AUTHORIZATION OF BIOLOGICS IN THE EU

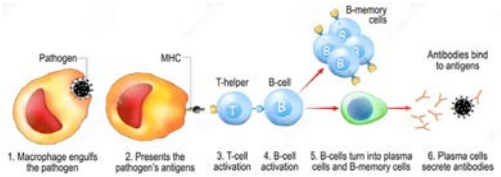
- in EU, all biologic medicinal products (both innovative and biosimilar) are authorized through the **centralized procedure**



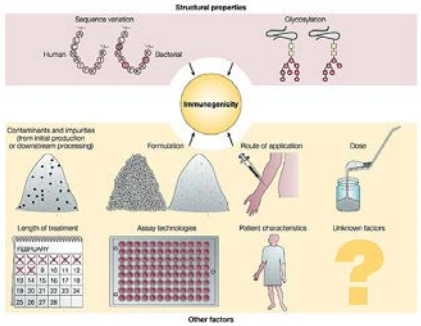
IMMUNOGENICITY OF BIOLOGICAL DRUGS

- proteins (especially foreign ones) make good antigens
- most therapeutic proteins are **immunogenic**
- anti-drug antibodies are typically responsible for **increased clearance** (**affect PK**) – loss of efficacy over time
- some anti-drug antibodies are **neutralizing** (**affect PD**) and can even cross-react with endogenous target (**affect safety**)

https://www.dreamstime.com/immune-response-humoral-immunity-stages-vector-illustration-image212834413



IMMUNOGENICITY OF BIOLOGICAL DRUGS



Shellekens H. *Nat Rev Drug Disc* 2002

Immunogenicity is affected by:

- degree of divergence from human protein sequence
- choice of expression system (e.g. glycosylation profile)
- contaminants (e.g. HCPs, LPS, CpG)
- drug's intrinsic activity
- formulation
- application route
- therapeutic regime
- drug product storage conditions
- patient genetics, etc.

FIRST GENERATION BIOPHARMACEUTICALS

- simple replacement proteins
- identical AA sequence to that of endogenous protein

- insulin
- growth hormone
- interferon α
- interferon β
- filgrastim (G-CSF)
- sargramostim (GM-CSF)
- epoetin α
- epoetin β
- follitropin α
- follitropin β
- lutropin α
- glucagon
- calcitonin
- anakinra (IL1-ra)
- alteplase (t-PA)
- streptokinase

+ many others

SECOND GENERATION BIOPHARMACEUTICALS

- **tailored PK and/or PD properties**
- **protein engineering** at the level of cognate gene
- **metabolic glycoengineering**

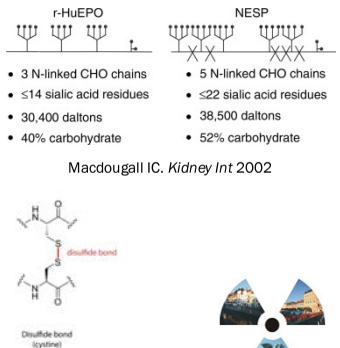
- humanization of antibodies
- antibody fragments
- fusion proteins
- minor modification of AA sequence
- modified glycosylation
- acylated proteins
- PEGylated proteins

extended/shortened half-life
improved solubility
improved (metabolic) stability
improved potency
reduced immunogenicity
conversion to antagonists
new functions

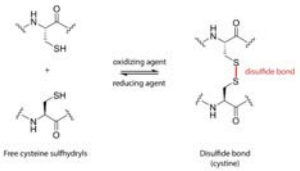
SECOND GENERATION BIOPHARMACEUTICALS - EXAMPLES

AMINO ACID ENGINEERING

- typically small changes of the AA sequence
- introduction of glycosylation sites (darbepoetin α = NESP) → extended plasma half-life ($t_{½}$)
- removal of cysteines (aldesleukin) → prevention of dimer formation



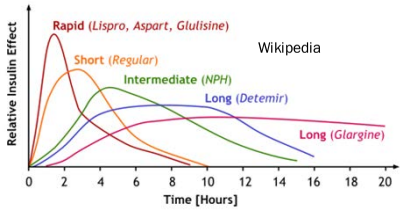
Macdougall IC. *Kidney Int* 2002



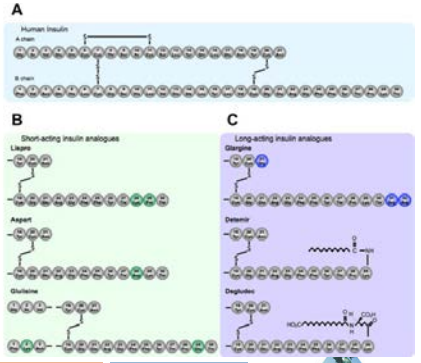
SECOND GENERATION BIOPHARMACEUTICALS - EXAMPLES

AMINO ACID ENGINEERING

- modulation of insulins' PK



Wikipedia

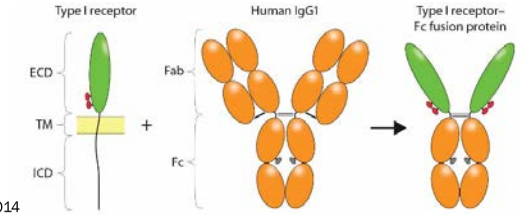


Borgono C & Zinan B. *Endocrinol Metab Clin North Am* 2012

SECOND GENERATION BIOPHARMACEUTICALS - EXAMPLES

FUSION PROTEINS

- **chimeric** proteins made from **fusion genes** created by joining parts of two different genes
- translated as a **single polypeptide sequence**
- fusion of extracellular receptor domains to antibody Fc region (e.g. etanercept) → dimeric structure, extended $t_{½}$, IgG effector functions

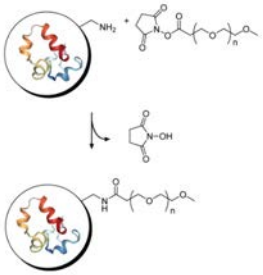


Linderholm AL & Chamow SM. *Bioprocess Int* 2014

SECOND GENERATION BIOPHARMACEUTICALS - EXAMPLES

PEGYLATED PROTEINS

- **conjugation with polyethylene glycol (PEG)**
- improved solubility
- reduced immunogenicity
- limited glomerular filtration
- extended $t_{½}$
- e.g. CERA (PEG-epoetin β), PEG-interferon α2a/b, certolizumab pegol, pegvisomant, pegfilgrastim

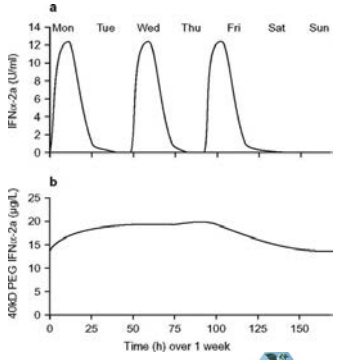


https://broadpharm.com/blog/pegylation-and-pegylation-reagents

SECOND GENERATION BIOPHARMACEUTICALS - EXAMPLES

PEGYLATED PROTEINS

	Protein →	PEG-Protein
In vitro activity:	100 %	reduced
In vivo activity:	100 %	enhanced

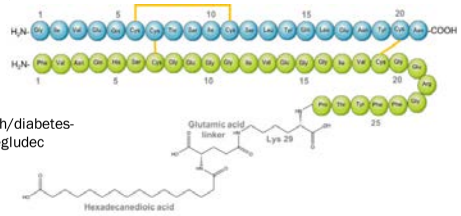


Kozlowski A et al. *BioDrugs* 2001

SECOND GENERATION BIOPHARMACEUTICALS - EXAMPLES

ACYLATED PROTEINS

- conjugation with fatty acids
- extended $t_{½}$ due to albumin binding
- e.g. insulins degludec & icodec, liraglutide (GLP-1-analog)

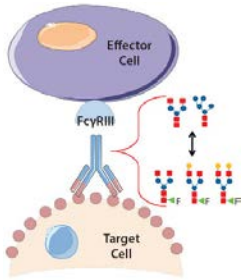


https://pdb101.rcsb.org/global-health/diabetes-mellitus/drugs/insulin/insulin-degludec

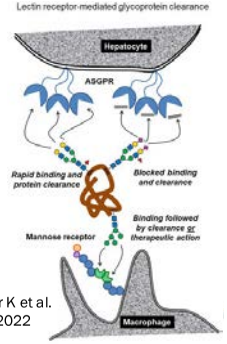
SECOND GENERATION BIOPHARMACEUTICALS - EXAMPLES

GLYCOENGINEERING

- modulation of glycosylation network in production cell line
- extended $t_{½}$
- increased potency of afucosylated antibodies



Ho DT et al. *BioProcess Int* 2016

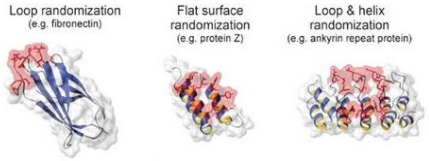


Dammen-Brower K et al. *Front Chem* 2022

SECOND GENERATION BIOPHARMACEUTICALS - EXAMPLES

ALTERNATIVE SCAFFOLD PROTEINS

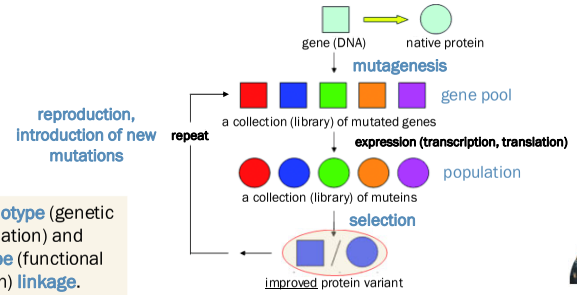
- ***in vitro* evolved** binders from small single-domain proteins
- cannot be designed: **surface randomization & library screening**
- easily produced in *E. coli*
- short $t_{½}$ & high specificity → <u>of interest as radioimaging agents</u>



Binz HK et al. *Nat Biotechnol* 2005

MOLECULAR EVOLUTION *IN VITRO*: A DIRECTED EVOLUTION

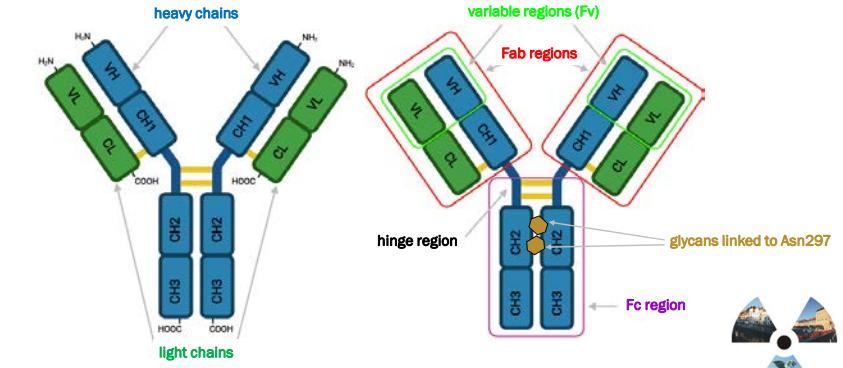
Mimicking natural evolutionary principles to improve proteins (i.e. endow them with new functions).



Direct **genotype** (genetic information) and **phenotype** (functional protein) **linkage**.

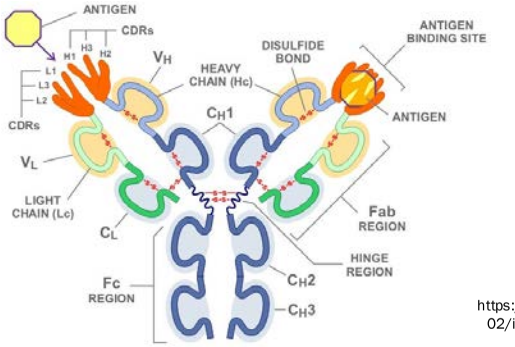
MONOCLONAL ANTIBODIES

IMMUNOGLOBULIN G (IgG)

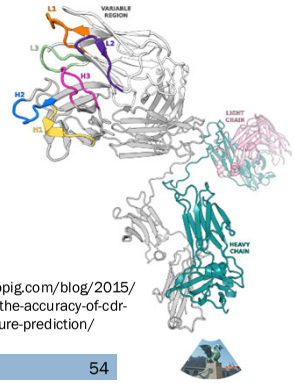


IMMUNOGLOBULIN G (IgG)

Abs' antigen binding sites (**paratopes**) are highly specific due to preorganized structure of 6 loops termed **CDRs** (**complementarity determining regions**) – 3 in each of the variable domains.



https://teaching.ncl.ac.uk/bms/wiki/index.php/Antibody



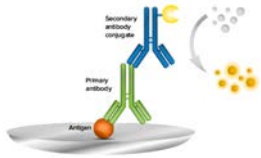
https://www.blopig.com/blog/2015/02/improving-the-accuracy-of-cdr-h3-structure-prediction/

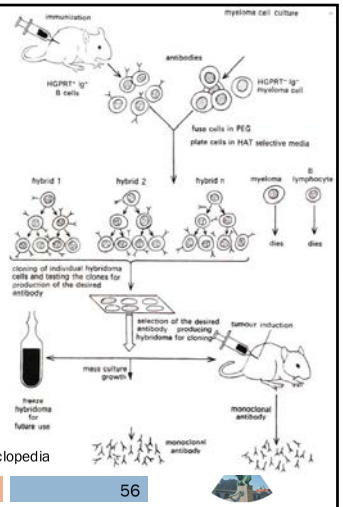
HYBRIDOMA TECHNOLOGY

- in 1975 Köhler and Milstein devised a method to produce murine **monoclonal antibodies (mAbs)**
- pro:
  - first reliable source of mAbs
  - available in large quantities
  - adequate for diagnostic purposes
- contra:
  - short $t_{½}$
  - highly immunogenic
  - insufficient activation of effector functions in humans

HYBRIDOMA TECHNOLOGY

- a mouse is immunized
- splenocytes are isolated and fused with immortal myeloma cells
- hybridomas are grown in selection medium
- individual cell clones are screened and expanded



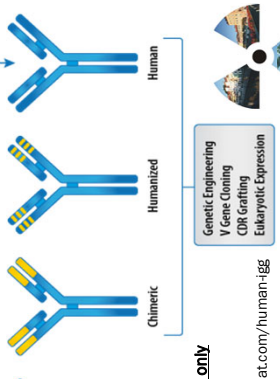


Biocyclopedia

HUMANIZATION OF mAbs

chronic therapy possible

- based on fusion genes
- **chimeric mAbs**: grafting of mouse variable regions (VH & VL) onto human IgG scaffold
- **humanized mAbs**: grafting of mouse CDRs onto human IgG scaffold
- significant decrease in immunogenicity
- improved effector functions

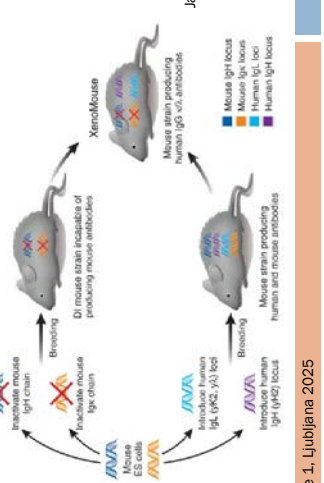


acute intervention only

https://www.biocat.com/human-igg

FULLY HUMAN mAbs: XenoMouse

- developed through hybridoma technology using transgenic mice harboring human IgG genes



Jakobovits A et al. *Nat Biotechnol* 2007

ANTIBODY PHARMACOKINETICS

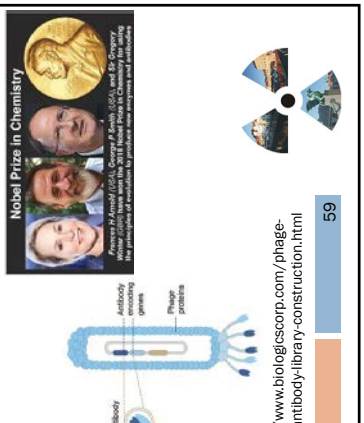
- mAbs are typically administered **parenterally** (typically i.v. or s.c., some i.m.)
- upon s.c. or i.m. administration mAbs enter the lymph and from thereon blood
- from blood mAbs are distributed via **convection** (due to pressure gradient)
- **poor penetration** into tissues due to large size
- mAbs bound to receptors (FcRy on immune cells, lectin receptors on hepatocytes or antigens on target tissue (**target-mediated drug disposition**)) get internalized and are degraded...

BUT

- ...there is a **recycling mechanism**

FULLY HUMAN mAbs: PHAGE DISPLAY TECHNOLOGY

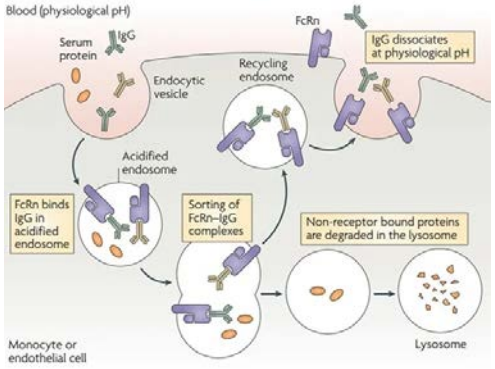
- developed through ***in vitro* molecular evolution** (Ab fragment library screening)



https://www.biologicscorp.com/phage-display-antibody-library-construction.html

ANTIBODY PHARMACOKINETICS

- $t_{½}$ is much longer compared to other proteins of similar size:
  - human mAb: 14-21 days
  - chimeric mAb: 8-10 days
  - mouse mAb: 30-40 hours
- $t_{½}$ is also dependent on mAb's isoelectric point (longer in negatively charged mAbs due to electrostatic repulsion from cell surface)



Roopenian DC & Akilesh S. *Nat Rev Immunol* 2007

ANTIBODY PHARMACODYNAMICS

- mAbs display a range of distinct mechanisms of action
- effector functions are dependent on the Fc region/IgG subclass
- often multiple mechanisms are responsible for the therapeutic effect
- some antibodies have several therapeutic indications

Properties	IgG1	IgG2	IgG3	IgG4
Approximate molecular weight (kDa)	146	146	165	146
Hinge length (number of amino acids)	15	12	62	12
Antibody-dependent cell-mediated cytotoxicity	+++	+/−−	++	+/−−
Antibody-dependent cell-mediated phagocytosis	+	+	+	+/−−
C1q binding	+	+/−	+++	−
Complement-mediated cytotoxicity	++	+/−	++	−
FcRn binding	+	+	+/−	+
Plasma half-life (days)	21	21	5–7.5	21
Approximate average plasma concentration (mg ml^{-1})	9	3	1	0.5

Almagro JC et al. *Front Immunol* 2018

ANTIBODY PHARMACODYNAMICS

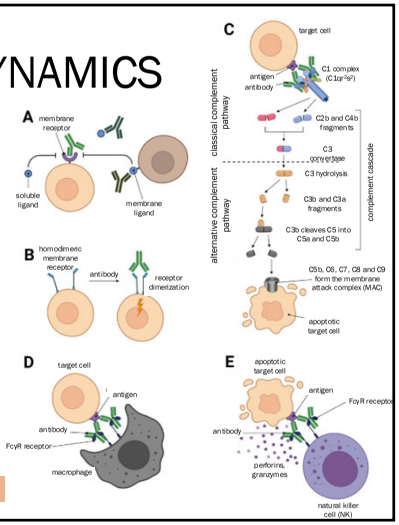
A. **receptor antagonism/downregulation**
B. **receptor agonism**
C. **complement-dependent cytotoxicity (CDC)**
D. **Ab-dependent cell phagocytosis (ADCP)**
E. **Ab-dependent cellular cytotoxicity (ADCC)**

+

targeted toxin (antibody-drug conjugates, ADCs)

or

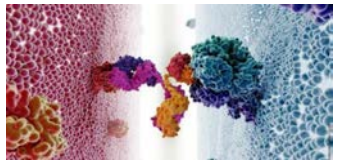
radionuclide delivery



BISPECIFIC ANTIBODIES

- have two different antigen-binding sites
- engage two different antigens and link them together, such as:
  - A. CD3 on T lymphocyte and TAA (EpCAM) on target cell (catumaxumab)
  - B. coagulation factors fX and fIXa (emicizumab)

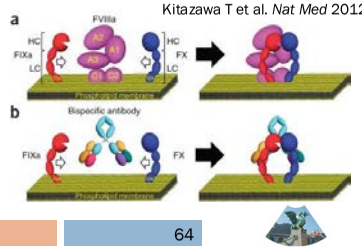
A.



https://www.mdanderson.org/cancerwise/bispecifics-antibodies--the-next-big-thing-in-lymphoma-treatment.h00-159540534.html

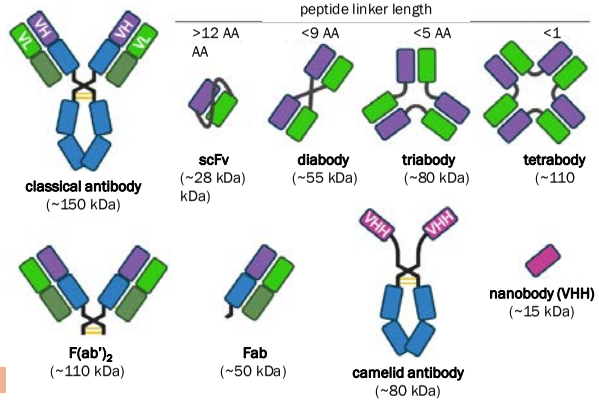
B.

Kitazawa T et al. *Nat Med* 2012



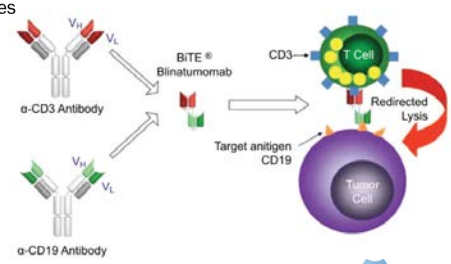
ANTIBODY FRAGMENTS

- same affinity
- same specificity
- smaller size → better tissue penetration
- reduced $t_{1/2}$ compared to full size mAbs
- compatible with expression in prokaryotic hosts

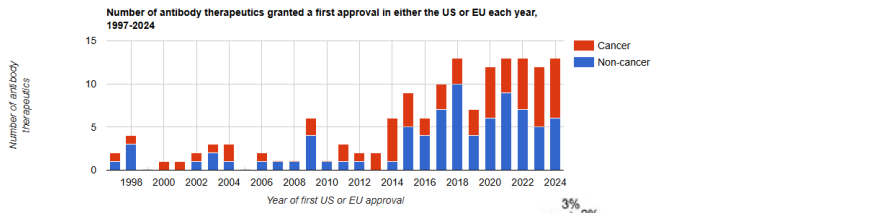


BISPECIFIC ANTIBODY FRAGMENTS

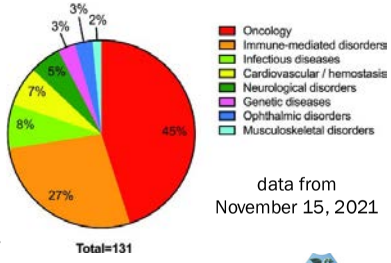
- bispecific T cell engagers (BiTEs) – tandem scFvs
- one arm binds to a tumor-associated antigen
- the other arm is directed to CD3 on T lymphocytes
- e.g. blinatumomab



https://www.biochempeg.com/article/252.html

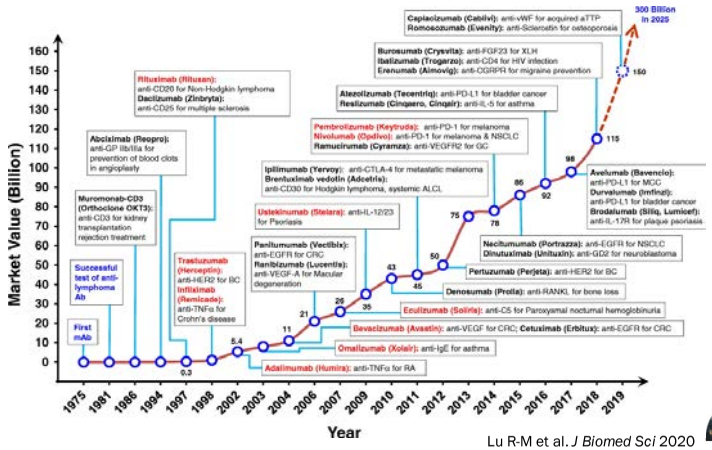


https://www.antibodysociety.org/resources/approved-antibodies/



data from November 15, 2021

Kaplon H et al. *Mabs* 2023



Lu R-M et al. *J Biomed Sci* 2020

SYNTHETIC PEPTIDE DRUGS

Typically fewer than 40 amino acid residues.

PEPTIDE DRUGS: PROS

- peptide drugs are considered biologics regardless of production method (synthetic vs. recombinant)
- **high potency, good specificity**
- good toxicological profile (safe)
- affordable
- small
- **good tissue penetration**
- a range of chemical modifications are possible (amidation, cyclization, incorporation of non-proteinogenic amino acids, acylation etc.)
- can address difficult/poorly druggable targets (e.g. PPI inhibitors)

PEPTIDE DRUGS: CONS

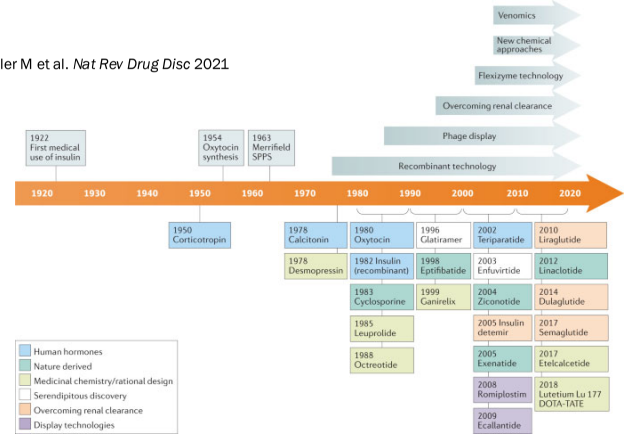
- typically administered parenterally
- targets are extracellular
- **extremely short $t_{½}$** due to extensive glomerular filtration
- metabolically unstable
- prone to denaturation, aggregation
- potentially immunogenic

S	**Strengths** • Good efficacy, safety, and tolerability • High selectivity and potency • Predictable metabolism • Shorter time to market • Lower attrition rates • Standard synthetic protocols	**W**	**Weaknesses** • Chemically and physically instable • Prone to hydrolysis and oxidation • Tendency for aggregation • Short half-life and fast elimination • Usually not orally available • Low membrane permeability
O	**Opportunities** • Discovery of new peptides, including protein fragmentation • Focused libaries and optimized designed sequences • Formulation development • Alternative delivery routes besides parental • Multifunctional peptides and conjugates	**T**	**Threats** • Immunogenicity • New advancements in genomics, proteonimics, and personalized medicine • Significant number of patent expiries • Price and reimbursement environment • Increasing safety and efficacy requirements for novel drugs

Drug Discovery Today

Fosgerau K & Hoffmann T. *Drug Disc Today* 2014

Muttenthaler M et al. *Nat Rev Drug Disc* 2021



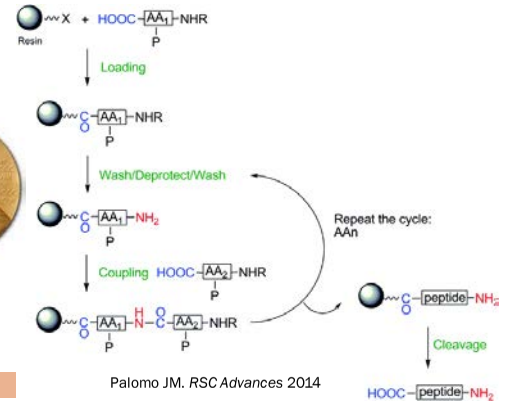
(SOLID PHASE) PEPTIDE SYNTHESIS

first (solution phase) synthesis of a peptide (oxytocin)

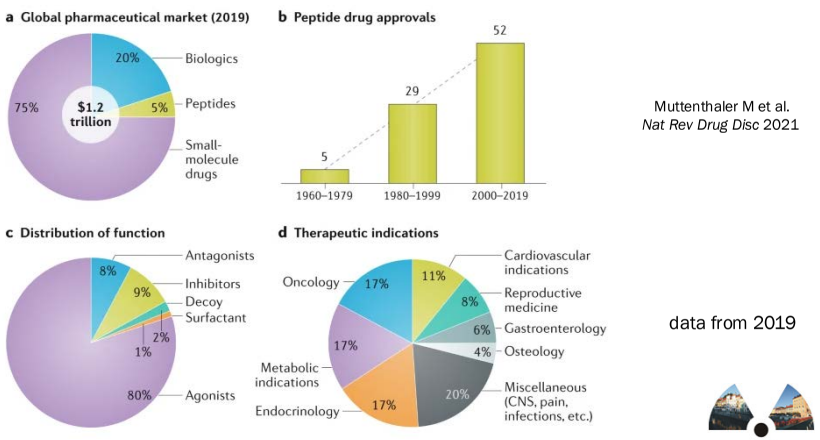
Vincent du Vigneaud
Nobel prize in chemistry 1955

development of solid phase peptide synthesis

Robert B. Merrifield
Nobel prize in chemistry 1984



Palomo JM. *RSC Advances* 2014

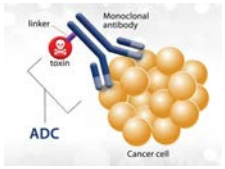


Muttenthaler M et al.
Nat Rev Drug Disc 2021

data from 2019

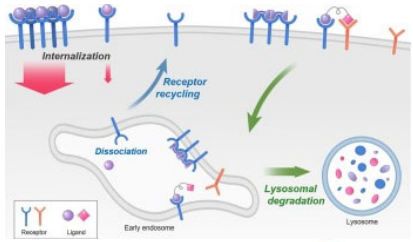
Generic name	Most common brand names	Companies	Indication	First approval	Sales in 2019 (US$ million)
Insulin and analogues	Ademelog, Apidra, Humulin, Humalog, Insuman, NovoMix, NovoRapid, Lantus, Levemir, Ryzodeg, Tresiba, Toujeo	Novo Nordisk, Eli Lilly, Sanofi	Diabetes	1982	25,000
Dulaglutide	Trulicity	Eli Lilly, Dainippon Sumitomo	Diabetes	2014	4,394
Liraglutide	Victoza, Saxenda	Novo Nordisk	Diabetes, obesity	2010	4,142
Leuprolide	Lupron, Eligard	AbbVie, Astellas, Takeda	Cancer	1985	2,022
Semaglutide	Ozempic, Rybelsus	Novo Nordisk	Diabetes, obesity	2017, 2019	1,694
Octreotide	Sandostatin	Novartis	Cancer	1988	1,585
Glatiramer	Copaxone, Glatopa	Teva, Sandoz	MS	1996	1,531
Teriparatide	Forteo	Eli Lilly	Osteoporosis	2002	1,405
Cyclosporine	Restasis	Allergan	Immune diseases, organ transplants	1983	1,189
Lanreotide	Somatuline	Ipsen	Acromegaly	2007	1,124
Carfilzomib	Kyprolis	Amgen	Multiple myeloma	2012	1,044
ACTH	Acthar	Mallinckrodt	IS, MS	1950	953
Linaclotide	Linzess, Constella	Allergan, Astellas Pharma	IBS-C	2012	877
Romiplostim	Nplate, Romiplate	Amgen, Kyowa Kirin	Chronic ITP	2008	841
Goserelin	Zoladex	AstraZeneca	Cancer	1989	813
Etelcalcetide	Parsabiv	Amgen, Ono Pharma	Hyperparathyroidism	2017	693
Exenatide	Byetta, Bydureon	AstraZeneca	Diabetes	2005	659
Teduglutide	Gattex, Revestive	Takeda	Short bowel syndrome	2012	555
Vasopressin	Vasostrict	Endo Pharma	CDI	NR	532

ANTIBODY/PEPTIDE-DRUG CONJUGATES



PAYLOAD DELIVERY

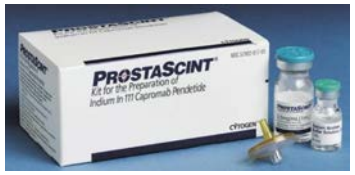
- selective ligand/Ab binding to surface receptors for payload delivery **to** and **into target cells**
- internalization via receptor-mediated endocytosis:
  - ligand binding strength (affinity)
  - valency of interaction
  - receptor clustering induced by ligand binding
  - receptor conformational changes upon ligand binding

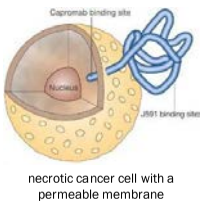


Sung Y et al. *J Control Rel* 2025

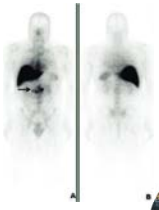
ANTIBODY/PEPTIDE LABELING FOR THERAPY OR IMAGING

- **imaging**: labeling with radionuclides for positron emission tomography (PET) or gamma camera scintigraphy (single-photon emission computed tomography, SPECT)
- *[^{111}In]-capromab pendetide*: anti-PSMA murine IgG1 for SPECT mapping of recurrent and metastatic prostate cancer





necrotic cancer cell with a permeable membrane

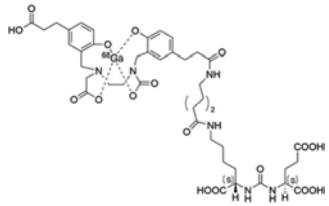


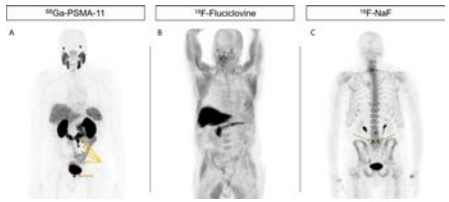
Bhargava P et al. *Am J Nucl Med Mol Imaging* 2016

ANTIBODY/PEPTIDE LABELING FOR THERAPY OR IMAGING

- **imaging**: labeling with radionuclides for positron emission tomography (PET) or gamma camera scintigraphy (single-photon emission computed tomography, SPECT)
- *[^{68}Ga]Ga-PSMA-11* (*[^{68}Ga]Ga-gozetotide*): Glu-urea-Lys-based PSMA ligand for PET imaging of prostate cancer

Ng TSC et al. *Front Oncol* 2021

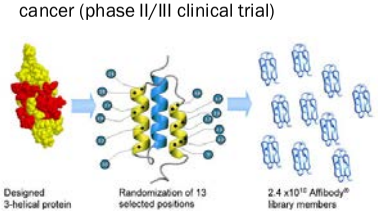


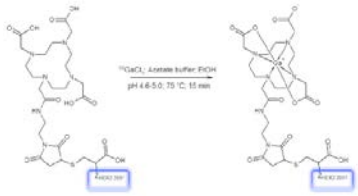


Carlucci G et al. *J Nucl Med* 2021

ANTIBODY/PEPTIDE LABELING FOR THERAPY OR IMAGING

- **imaging**: labeling with radionuclides for positron emission tomography (PET) or gamma camera scintigraphy (single-photon emission computed tomography, SPECT)
- *[^{68}Ga]Ga-ABY-025*: affibody for non-invasive PET mapping of HER2 expression in metastatic breast cancer (phase II/III clinical trial)





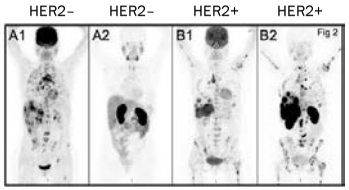
© Dolfe L 2011

Velikyan I et al. *Am J Nucl Med Mol Imaging* 2016

ANTIBODY/PEPTIDE LABELING FOR THERAPY OR IMAGING

- **imaging**: labeling with radionuclides for positron emission tomography (PET) or gamma camera scintigraphy (single-photon emission computed tomography, SPECT)
- *[^{68}Ga]Ga-ABY-025*: affibody for non-invasive PET mapping of HER2 expression in metastatic breast cancer (phase II/III clinical trial)

Sörensen J et al. *Theranostics* 2016

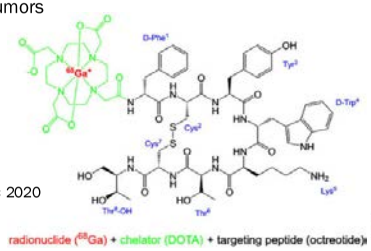


[^{18}F]FDG-PET	+	–	+	–
[^{68}Ga]ABY-025 PET	–	+	–	+

ANTIBODY/PEPTIDE LABELING FOR THERAPY OR IMAGING

- **imaging**: labeling with radionuclides for positron emission tomography (PET) or gamma camera scintigraphy (single-photon emission computed tomography, SPECT)
- *[^{68}Ga]Ga-DOTATOC* (*[^{68}Ga]-edotreotide*): synthetic somatostatin analog for imaging of SSTR-positive gastroenteropancreatic neuroendocrine tumors

Hennrich U & Benešova M. *Pharmaceuticals* 2020

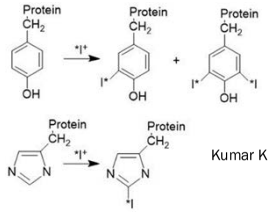


ANTIBODY/PEPTIDE LABELING FOR THERAPY OR IMAGING

- **targeted radiotherapy**: labeling with radionuclides for delivery of beta-minus radiation or alpha-particles
- systemic delivery of radiation to tumors while minimizing damage to surrounding tissues
- *[^{90}Y]-ibritumomab tiuxetan*: murine anti-CD20 IgG1 for targeted delivery of β-emitter yttrium-90 to B lymphocytes in lymphoma
- *[^{177}Lu]Lu-DOTATATE* (*[^{177}Lu]Lu-oxodotreotide*): somatostatin analog for targeted delivery of β-emitter lutetium-177 to SSTR2-overexp. tumors
- *[^{177}Lu]Lu-PSMA-617* (*[^{177}Lu]Lu-vipivotide tetraxetan*): Glu-urea-Lys-based PSMA ligand for targeted delivery of β-emitter lutetium-177 to PSMA-positive metastatic castration-resistant prostate cancer

BIOCONJUGATION CHEMISTRIES

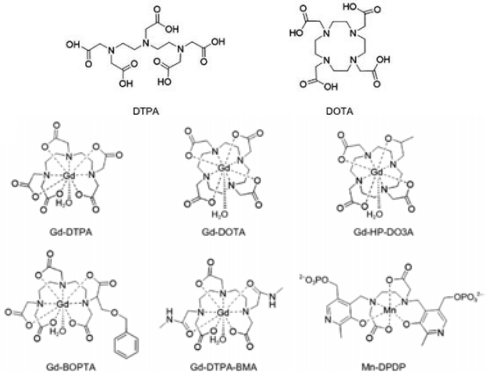
- nonmetallic radionuclides (^{123}I) introduced **directly** via electrophilic aromatic substitution on Tyr or His



Kumar K & Ghosh A. *Molecules* 2021

- radiometals (e.g., ^{68}Ga, ^{89}Zr, ^{111}In) introduced via **bifunctional chelating agents (BCAs)** bound to Lys or Cys (random vs. site-selective conjugation)

BIOCONJUGATION CHEMISTRIES



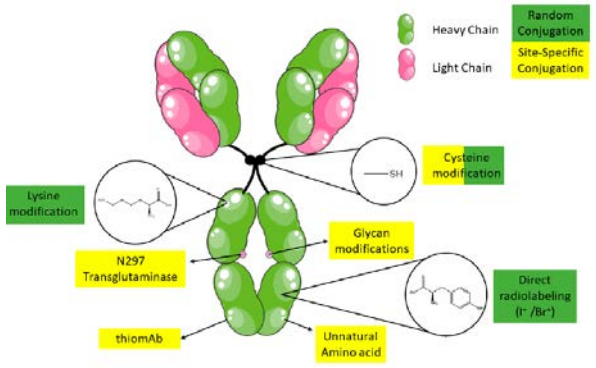
chelating agents

DOTA:
1,4,7,10-tetra-azacylcododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid

DTPA:
diethylenetriamine pentaacetic acid

Ding L. *Sci China Mat* 2018

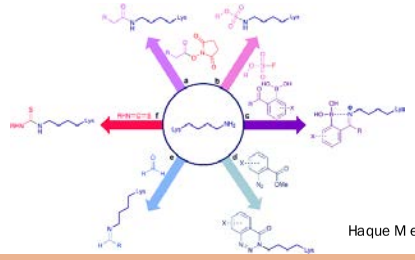
CONJUGATION CHEMISTRIES



Dewulf et al. *Cancers* 2020

SOLVENT ACCESSIBLE RESIDUES - Lys

- 30 Lys (out of 80) per IgG are solvent accessible
- ε-amino group is activated @ pH 9 (unprotonated)
- BCAs are coupled via activated esters, iso(thio)cyanates etc.

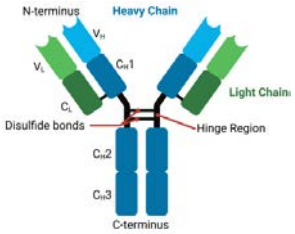


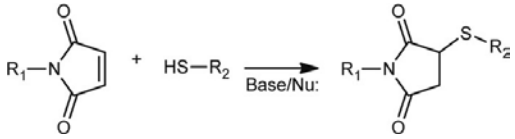
(a) NHS (*N*-hydroxysuccinimide) esters
(b) sulfonyl fluorides
(c) iminoboronates
(d) diazonium salts
(e) aldehydes
(f) isothiocyanates

Haque M et al. *Chem Commun* 2021

SOLVENT ACCESSIBLE RESIDUES - Cys

- 2-4 interchain disulfide bonds in the IgG hinge region are surface exposed
- reduced to form reactive thiols
- BCAs are coupled via maleimide groups (forming a thioether bond)

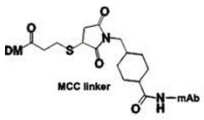


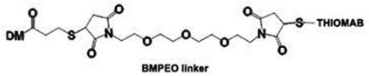


Wu D et al. *Nature* 2019

SOLVENT ACCESSIBLE RESIDUES - Cys

- **unpaired Cys** residues can be introduced in IgG sequence via **amino acid engineering** → site-specific conjugation
- e.g. 'thiotrastuzumab' (an engineered anti-HER2 mAb)



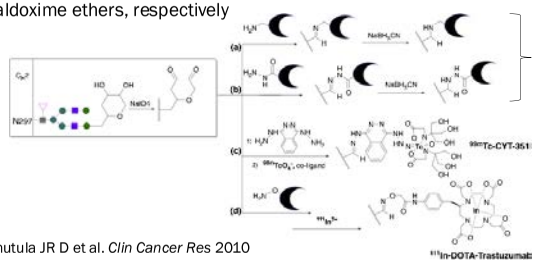


heterogeneity: - number of conjugate attached
- regioisomers
- batch-to-batch variability

Junutula JR D et al. *Clin Cancer Res* 2010

GLYCANS

- cis-glycol groups of glycans attached to Asn297 can be oxidized with periodate (IO4-) to yield aldehyde groups
- aldehydes react with amino, hydrazide, and aminoxy groups to form imides, hydrazones, and aldoxime ethers, respectively

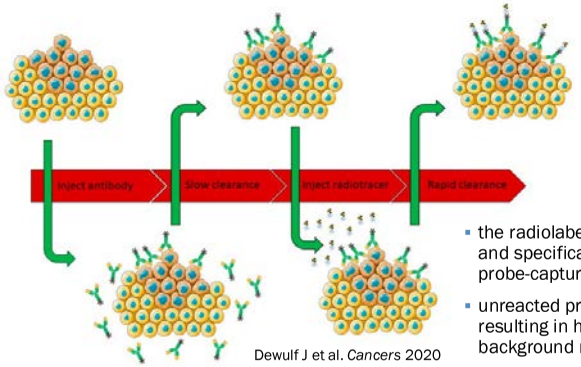


Junutula JR D et al. *Clin Cancer Res* 2010

PRE-TARGETING APPROACH

- circumvents limitations of directly radiolabeled mAbs (long $t_{½}$, poor tissue penetration → high radiation dose to healthy tissues)
- two-step approach:
  1) **mAb** (**conjugated to probe-capturing moiety**, e.g. avidin, or **bispecific mAb**) is allowed to accumulate in target tissue;
  2) **complementary small radiolabeled probe** (e.g. conjugated to biotin) is injected later

PRE-TARGETING APPROACH



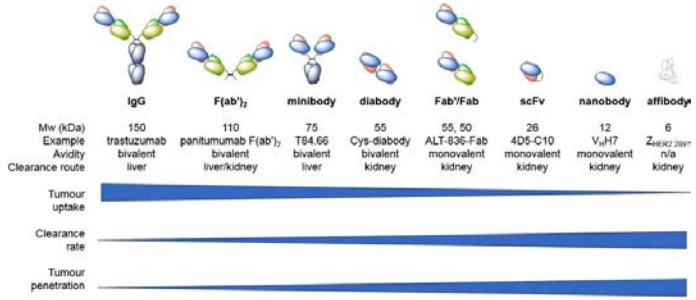
Dewulf J et al. *Cancers* 2020

Not always optimal:
long clearing times required for unbound mAb to be removed from circulation, during which the target tissue-bound mAb might get internalized.

- the radiolabeled probe 'finds' and specifically reacts with the probe-capturing moiety
- unreacted probe is cleared fast, resulting in high target-to-background ratio

ANTIBODY/PEPTIDE-DRUG CONJUGATES: THINGS TO CONSIDER

- targeting selectivity
- avidity
- immunogenicity
- clearance rate
- tissue penetration



Lin M et al. *Curr Probl Cancer* 2021

THANK YOU!

ANY QUESTIONS?

Methods for determining cell purity and viability

Janja Zupan graduated in 2005 at the Department of Clinical Biochemistry (supervisor prof. dr. Janja Marc) in the field of genetics of osteoporosis. Since November 2009 she has been working as a Teaching Assistant at the Faculty of Pharmacy, Department of Clinical Biochemistry. In 2011 she completed part of her research and PhD work at Institute of Genetics and Molecular Medicine, University of Edinburgh, UK. She finished her PhD entitled The influence of the proinflammatory cytokines on the RANK/RANKL/OPG in bone tissue of osteoporotic and osteoarthritic patients in 2012. From 2014-2016 she worked at the Institute of Biomedical Sciences, University of Aberdeen, UK at the Aberdeen Centre for Arthritis and Musculoskeletal Health, where she gained knowledge in mesenchymal stem cells and regenerative medicine. She finished this postdoctoral fellowship identifying a new population of synovium-derived mesenchymal stem cells (Nature Communications 2017). After having come back to her home university, she continues working on human mesenchymal stem cells and regenerative medicine with the aim of better understanding of their role in different degenerative disorders and different joint tissues.

Martina Gobec graduated at the Faculty of pharmacy, University of Ljubljana in 2008 and continued her postgraduate education as a young researcher, enrolled in the programme of Biomedicine, field Clinical biochemistry and laboratory biomedicine. In 2012, she successfully defended her doctoral thesis, which was focused on the evaluation of serine protease inhibitors as inducers of apoptosis. From 2013, she was employed as an assistant at the Department of clinical biochemistry, and in 2021 became an associated professor for Toxicological chemistry at the Faculty of pharmacy. Her research skills include vast knowledge in cell culturing (cultivation of suspension and adherent cells, isolation of cell populations) and extensive experience in various molecular techniques (spectrophotometry, electrophoresis, western blot, flow cytometry, immunoassays, viability, and cytotoxicity assays,…). Her current area of research is focused on the evaluation of targets in malignant diseases and the identification of compounds that modify the effects of selected targets (the focal points being the proteasome and the immunoproteasome). She published her research as a (co-)author in over 40 original scientific publications.

The lecture has the following learning objectives:

1. Students are able to choose a method to differentiate between living and dead cells.
2. Students are able to determine cell viability after staining with trypan blue.
3. Students are aware of different techniques to determine cell viability and critically evaluate their advantages and limitations.
4. Students understand the basics of flow cytometry.
5. Students are able to determine the ratio of different cell types in a sample after an analysis on the flow cytometer.

Cell counting, viability and purity

In vitro quality control is an essential part of the procedure of labelling white blood cells (WBCs). In this segment, we will address the methods available for determination of cell count, their viability and the purity of the WBCs.

Cell viability

Cell counting is a pivotal step prior in cell culture work. We need to determine the number of viable cells to quantify the number of cells needed for further processing, seeding, manipulation and cryopreservation. Cells are most commonly counted using a special microscopic slide with a 0.1 mm deep chamber called a haemocytometer. The chamber has imprinted a special microscopic grid as shown in Figure 1. The grid consists of nine 1x1 millimetre squares, separated from each other by a triple line. The four corner squares are further divided into 16 smaller squares, and the central square is divided into 25 squares (Figure 1).

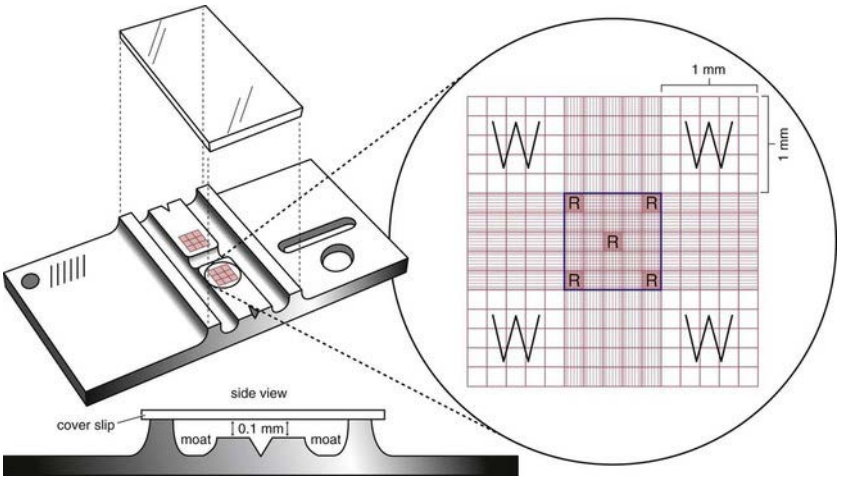


Figure 1. Haemocytometer and the grid.

When preparing the cells, the cell suspension should be dilute enough that each cell can be clearly distinguished and no two cells are clumped together. Ideally we want somewhere between 100 to 150 cells on each big corner square. If the cells are too close together and are overlapping, you can dilute the suspension further. If there are less than 50 cells per square, the cells need to be centrifuged and resuspended in a smaller volume of phosphate buffered saline (PBS). When counting the cells, we only count the cells that lie inside the large corner square and cells that lie on the outer grid lines of the square as shown on Figure 2B.

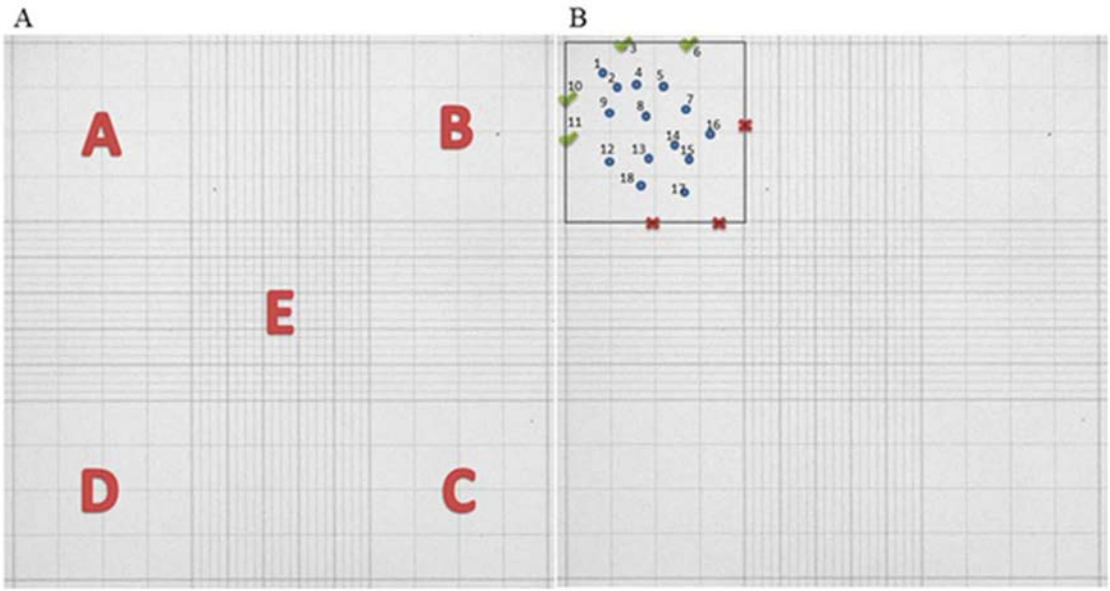


Figure 2. Counting cells on a haemocytometer.*When counting the cells we normally count the cells in the four large outer corners, marked A, B, C and D (A). If there are many cells, only the cells in the middle square E can be counted. Count the cells that lie inside the large corner square, and cells that lie on the outer grid lines of the square (B).*

The number of viable cells in 1 mL of the cell suspension can then be calculated using the following equation:

$$C\ (cells/mL) = \frac{number\ of\ living\ cells\ (all\ squares) \times dilution\ factor \times 10{,}000}{number\ of\ squares\ counted}$$

Cell death can occur through different mechanisms, ranging from regulated processes like programmed cell death (apoptosis) to rapid and uncontrolled events like necrosis. A cell is considered dead if it meets any of the following conditions:

- the integrity of the plasma membrane is compromised;
- the cell has broken into apoptotic bodies;
- fragments of the cell have been engulfed by neighbouring cells.

Dying cells not only lose their functionality but can also induce unfavourable reactions in nearby cells. Hence, it's crucial to ensure that the labelling of white blood cells (WBCs) doesn't impact their viability. Viability dyes are one of the most common means of assessing cell death and are either fluorescent or coloured molecules, which discriminate between living and dead cells**.** The most frequently used dyes include the so-called exclusion dyes, which cannot cross intact plasma membranes (e.g. propidium iodide) and thus label only dead cells. One of the most commonly used dye is Trypan blue, which capitalizes on the fact that viable cells possess intact membranes impermeable to the dye, whereas the

compromised membranes of dead cells permit the dye's entry, resulting in a blue stain. The cell viability is calculated as the ratio of live cells compared to all cells multiplied by 100.

$$viability\ (\%) = \frac{number\ of\ live\ cells}{total\ number\ of\ cells}\ x\ 100$$

Staining with Trypan blue can be performed manually or automatically. However, the analysis is performed on a small number of cells and therefore data can be lost. Analogous to this assay is staining with a fluorescent excluding dye and performing the analysis on a flow cytometer. In this method, thousands of cells are analysed and thus provide statistically more relevant data.

Cell recovery test (purity)

The test consists of counting the cells that are obtained during different phases of the cell separation and labelling process (i.e. after the centrifugation steps and at the end of procedure) to verify that red blood cell and platelet contaminations are within an acceptable range (Table 1).

Table 1. Acceptable limits for blood cell types during the preparation of labelled white blood cells from blood sample to injection of labelled cells (range)

	Basal (in peripheral blood)	In serum (after RBC sedimentation)	Labelled cells ready to be injected	Other cell type to WBC ratio
WBC (10^3/mm^3)	4–15	30–150	70–350	
Granulocytes (%)	45–95	60–100	80–100	
Lymphocytes (%)	10–45	5–20	2–15	
RBC (10^6/mm^3)	4–6	0.05–0.5	0.05–0.5	<3
Platelets (10^3/mm^3)	140–400	50–200	50–200	<1

RBC-red blood cells; WBC - white blood cells