Tutorial 1 – Acyclovir, an antiviral agent

1. Describe the life cycle of a virus, explaining its parasitic nature in relation to the host cell.
2. Discuss the general strategy of designing drugs which target infectious agents but not the patient’s own cells, and identify stages in the life cycle of viruses which could potentially allow therapeutic intervention.
3. Explain the specific strategy of using Acyclovir and other nucleoside analogues as antiviral drugs.

- Viruses are obligate parasites, with genetic material in the form of DNA/RNA. They have no organelles and are not cells – they cannot reproduce outside of cells.
- There are small and large virii, relating to amount of genetic material they have, and so the amount of virus-specific genes. Thus, the bigger the virus, the more proteins that differ from the host and the more potential targets for drug intervention and hence therapy against that virus.
- Use an example like Herpes simplex to describe the life-cycle of a virus. This can be used to find biochemical processes that might feasibly lend themselves to drug interaction – e.g. can we lend them to drug intervention, prevention of genomic incorporation, viral DNA replication or possibly viral production and packaging prevention.
- One should appreciate the concept of drugs that are structural analogues of naturally occurring molecules in cells and how these drugs may be used to fight against a virus and not the host. The basic strategy is the relation of protein structure to function – we take advantage of the difference between viral and human protein isoforms and utilise them to perturb key viral life cycle processes without affecting the patient.
- Here, Herpes has its’ own codes for thymidine kinase and DNApol – there are host versions of these too, but there are sufficient differences between substrate specificities and activities, allowing manipulation without greatly affecting cellular DNA synthesis.
- Acyclovir and related nucleotide and nucleoside analogues are effectively biochemical confidence tricks whereby the viral thymidine kinase has a high affinity for acycloGMP and hence generates large amounts of acycloGTP in infected cells. This in turn is an excellent substrate for the viral DNApol and so is incorporated into the growing DNA strand of viral DNA.
- Unfortunately, acycloGTP blocks further DNA polymerisation due to no 3’OH group being available for the next phosphate bond to be created, thus blocking viral replication. On the other hand, the acycloGTP is a poor substrate for host cell DNApol so is less incorporated, sparing the cell the brunt of the drug’s effect.
- Acyclovir is not a cure for the infection, but greatly suppresses it by reducing the overall viral load, allowing the host’s immune system to then work to eradicate infection completely. Of course, there are some situations in which this does not occur such as in immunocompromised and HIV patients, so here therapy is continuous and mandatory – many such patients will commonly have Herpes and cytomegalovirus.
- Acyclovir isn’t just for the immunosuppressed but is also sold OTC for Herpes cold sore treatment (Zovirax) and POM for Varicella zoster infection (Chicken Pox).

Tutorial 2 – Creatine kinase and myocardial infarction

1. Describe the localisation of the following substances in terms of whether they are more concentrated on the inside or outside of a living cell: ions, sugars and other small organic molecules, enzymes and other proteins, nucleic acids.
2. Explain the structures and processes which allow this localisation to be maintained in living cells, and predict the consequences of cells dying.
3. Discuss using muscle as an example why different specialised cell types may contain different enzymes and isoforms of enzymes.
4. Explain the diagnostic use of blood creatine kinase measurements for myocardial infarctions.

- A MI is the death of heart muscles, with cells dying due to oxygen deprivation. This is due to blockage of the coronary arteries (link atherosclerosis and risk factors for early development).
• Cell contents are released upon death due to proteins held against conc. Gradients being released into serum. Therefore the levels of such proteins including CK and lactate dehydrogenase can be used as indirect indicators of cell death.
• CK is normally present in all cells at very low levels, but is high in metabolically active cells such as the brain, heart and skeletal muscle.
• CK can be specifically related to MI by its structure. CK is a protein made up of two subunits or monomers to form a dimer. These two monomers are coded for by two different genes, forming a B and an M isoform. These two have the same molecular weight (43kDa) but differ in their pl (B=6.77, M=5.34). Monomers associate and bind to one another in the cytoplasm to produce active dimers. Thus 3 configurations are possible-BB, BM and MM.
  o The brain only expresses B, and skeletal muscle M, so they only have BB and MM present in their cells respectively.
  o Cardiac tissue is the only type that has the hybrid BM (70% MM, 30% MB) form, so cardiac fibre death is related to CK-BM isoform serum presence.
• The serum levels are directly proportional to the amount of cardiac cell death. Each monocyte can be considered to have approximately the same volume and hence a equal likelihood of dying independent of size. Each cell’s death releases a quantum of CK into extracellular fluid -> serum. CK serum peaks at a 24h post MI.
• Thus, simple CK serum activity is not a sufficiently good diagnostic indicator of cardiac cell death, as this could be for any of the 3 tissues so we must test for the BM form. We might be able to use gel electrophoresis or column chromatography to separate on charge and weight but these are slow and require technical assistance which is not suitable in a busy Casualty department.
  o We could use antibodies, but none have been developed solely for BM interaction. An BB form antibody does exist though, so we can use it to determine the remaining reaction, potentially giving the reaction for both BM and MM forms is present.
• This test is given in a battery of others so is used to ascertain infarct size and timing (via level changes) as opposed to MI detection. Other markers for MI damage include Serum oxaloacetate transaminase (SGOT), lactate dehydrogenase (LDH) and cardiac troponin. Troponin is a Ca²⁺ switch in muscle and Cardiac troponins I & T are tissue specific. Thus their presence in the serum indicates MI (usually 48h post Mi and persisting for approx. 5 days).

Tutorial 3 – Metabolic poisons

1. Explain the effect of an uncoupler (e.g. dinitrophenol or DNOC), a cytochrome oxidase inhibitor (e.g. cyanide or carbon monoxide) and an ATP synthase inhibitor (e.g. oligomycin) on the respiratory rate of mitochondria.
2. Discuss the symptoms that might result in a patient poisoned by one of these substances.

• ATP is not made in a cell unless ATP is required (balance of supply and demand). The fundamental cellular structures/processes are the mitochondrion and the Kreb’s Cycle/Ox. Phos. they house. Mitochondrial respiration is dependent upon ATP due to physio-chemical coupling.
  o As substrates move along the e⁻ transfer chain, each component of the complex uses the energy released in the electron transfer to transport H⁺ across the inner mitochondrial membrane, so the [H⁺] is higher outside.
  o This is utilised by ATP synthase to drive the ratchet action of the protein head. This “metabolic coupling” of the ETC and Ox. Phos. ensures that substrates are only metabolised when there is a demand for ATP. Oxygen consumption at the end of the chain only occurs in the presence of substrate and ADP.
• The rate of resp. is higher in succinate/ADP presence than glutamate/malate/ADP due to proton differences. NADH-linked substrates such as malate feed into the ETC via NADH dehydrogenase (complex I), so can generate 3 ATP per oxygen molecule reduced to water.
• Contrastingly, succinate oxidation feeds electrons into the chain via FADH₂ and succinate dehydrogenase (complex II) thus utilising two phosphorylation sites. So to generate a given amount of ATP, more succinate must be oxidised than glutamate/malate so more oxygen used.
**NB-why do glu/mal need simultaneous addition?** In vivo, these 2 substrates are required for the mal/asp shuttle: the principal mechanism for the movement of reducing equivalents (NADH during glycolysis) from the cytoplasm to the mit. Matrix. The [oxaloacetate] in mit. In vivo is exceedingly low (0.1µM) whereas [malate] is 10mM, due to the unfavourable reaction catalyzed by malate dehydrogenase.

- In the experiments involving isolated mit. The addition of malate alone causes the build-up of oxaloacetate, which would inhibit the malate dehydrogenase and therefore NADH production (which is the e- source for the respiratory ETC).
- However, the addition of glutamate allows the excessive oxaloacetate to be removed from the mit. Matrix via the mal/asp shuttle, and therefore the production of NADH to continue while malate available. Moreover, an active mal/asp shuttle allows for α-ketoglutarate to be produced in the mit. Matrix making it available for its exchange for malate by the malate/α-ketoglutarate antiporter, allowing a constant flow of malate into the mitochondria.

- When KCN is added, it reacts covalently with Fe³⁺ in cytochrome oxidase, inhibiting the terminal step of the ETC and so respiration ceases. Normally, oligomycin addition interferes with ATPsynthase, reducing its ability to utilise the proton gradient. In coupled mitochondria, this leads to respiration inhibition.
- In the tutorial case, the lack of effect indicates DNOC has uncoupled the mitochondria. Thus, Kreb’s cycle and Ox. Phos. Are running maximally but the link to ATP generation is broken free do substrate-released energy is lost as heat. This is consistent with large increases in mitochondrial oxygen consumption after DNOC addition.
- The metabolic effect of DNOC is that it, along with similar aromatic weak acids like dinitrophenol, are thought to pass readily across the mitochondrial inner membrane in their undissociated form and thus dissipating the proton gradient, disabling gradient formation. DNOC was used as a weight-loss drug, but was abandoned due to its fine therapeutic index and high ability for build-up in adipose tissue. It acts as a pesticide by uncoupling mit. Resp. so that the ETC runs uncontrollably and unproductively, so large amounts of metabolic fuel are consumed with the released energy being wasted as heat rather than harnessed.

- **Post-mortem features:** The principal fuels for this uncontrolled respiration are FA’s from the triglycerides stored in the adipose tissue, thus depleting the body’s fat stores. The accompanying excessive oxygen consumption leads to tissue hypoxia which the body attempts to alleviate by increased pulmonary respiration and erythropoesis in bone marrow.
  - Early rigor mortis can be explained by considering muscle biochemistry. The power stroke moves actin filaments relative to the heads of myosin, so shortening the muscle fibres. This involves ATP hydrolysis to ADP by myosin ATPase. To relax the fibre for the next power stoke, the ADP must be replaced by ATP. As DNOC greatly increases ADP concentration, the contractile system is left in rigor.

**Tutorial 4 – Osteogenesis imperfecta: brittle bones or battered baby**

1. **Describe the molecular architecture of the collagen molecule and how this may be affected in osteogenesis imperfecta.**

2. **Use the genetic code to deduce the nature of mutations within the collagen 1A1 gene, predict their effect on electrophoresis of the protein, and explain the biochemical consequences of mutations on the assembly of type I collagen and hence on bone structure and strength.**

3. **Explain the basis of a "dominant negative" disorder and explain why heterozygotes are affected by osteogenesis imperfecta.**

There are a number of human diseases in which there are inherited defects in the structure of particular ECM molecules, with consequences for connective tissues—The best example being those that affect collagen. Collagen is an ECM protein synthesised by and secreted from a variety of cells, such as fibroblasts, and organised into insoluble fibres. These fibres are a major part of the ECM surrounding cells and giving mechanical strength and rigidity to tissues and organs. In particular they provide the tensile strength of skeletal tissues including bone, cartilage, tendons and ligaments. There are at least 5 major types of collagen which occur in different tissues. Although these
have distinct properties they all have the same triple helix structure which is the special feature of collagen. Associated with this is the unusual AA composition with its high [glycine]. Glycine is the smallest of the AA’s and occurs at every 3rd position in collagen where it faces the interior of the helix. Other features include the presence of the modified AA’s hydroxyproline and hydroxylysine.

OI- The main feature of this disease is repeated Fx of the long bones, and for this reason it can be misdiagnosed as child abuse. There are also malformed bones, with a whole range of genetic disorders leading to the disease. In the example used here, the defect is a point mutation (G→T), in the gene for collagen type I. This results in the substitution of the normal glycine residue with cystiene. The larger AA in the mutant molecule will cause stearic hindrance which generates kink in the normally straight triple helix, with a resulting defect in the assembly into fibres. Most cases of OI result from mutations in the glycine residues producing defective structural assembly. In this case, the AA cystiene has a reactive sulphydral group in its side chain. Thus not only is formation of the collagen triple helix disrupted but there can also be inappropriate S-S bonds between the two α1(I) chains in the helix. The resulting crosslinked polypeptide chains will migrate much more slowly than the individual chains when examined by gel electrophoresis in the presence of SDS (detergent). However in the presence of 2-mercaptoethanol the S-S bonds will be cleaved, allowing the chains to migrate according to their M.

The patient in this case is heterozygous, so if only one of her α1(I) chains will be abnormal while the other allele is making the normal version. In principle 50% of the chains will be normal and the other 50% abnormal, though in practise this exact ration rarely occurs in real genetic diseases. There may be some differences in the rate of transcription of the gene, rate of translation, mRNA stability or stability of protein formed leading to differing ratios. Because the collagen triple helix contains two α1(I) chains and will be disrupted if only one is the mutant form, the majority of the collagen fibres will be affected leading to a dominant phenotype. The major consequence of this is in the formation of bone, which is formed by the laying down of hydroxyapatite (a form of calcium phosphate) on the ordered scaffold of collagen-I. The abnormal collagen structure leads to defects in this mineralisation process, so that the pt. ends up with skeletal abnormalities and generally weak bones. Other problems that commonly occur with OI include eye, teeth, skin and ear abnormalities.

In the case presented, the patient was investigated by the direct study of her collagen protein. This would not be a suitable approach for prenatal diagnosis since sampling of foetal collagen would be impractical and risky. A better approach would be genetic screening of the foetal DNA obtained by chorionic villus sampling or amniocentesis, and amplified by PCR. Specific probes could be used to which were complementary to the DNA sequence which is known to site the mutation. Under the right conditions of temperature and ionic strength, the probe will only hybridise (bind to DNA) if it has the exact complementary sequence, enabling normal and mutant genes to be distinguished. Alternatively if the mutation altered a restriction enzyme recognition site, that would allow identification of normal and mutated DNA since only one would be cleaved by the enzyme to shorter fragments. Both methods rely on the mutation being one that is already known, but that could be checked on other family members.

From this tutorial, you should review 2° structure of proteins, types of bonds and distinguish between α-helix and collagen triple helix. The role of signal sequences, post-translation modifications including glycosylation, hydroxylation, proteolytic processing and cross-linking reactions, and distinguish the roles of different organelles. How to read off AA sequences from a gene sequence, initiating codon, exons and introns. Hybridisation between complementary pieces of DNA, and the reason for its dependency on temp. & ionic strength, and the ways in which gel electrophoresis can be used to distinguish protein isoforms.

**Tutorial 5 – Anaphylactic reaction**

1. Describe the progress of an immune response to an antigen, including switching of immunoglobulin class.
2. Explain the symptoms of an anaphylactic reaction in terms of antibody type, mediating cell type, secreted signalling molecules, and target tissues.

Anaphylaxis is an acute (immediate) type I hypersensitivity reaction that can be systemic, and is sometimes so overwhelming that it is life-threatening. It results from an IgE-mediated response to an allergen that is present throughout the body. The IgE response is thought to be important in defence against certain parasitic infection (eg. Nematodes). However, it can occur inappropriately to give rise to allergic reaction. The normal pathway of
lymphocyte activation occurs when antigen binds to surface IgM on lymphocytes to promote proliferation and secretion of antibody. Usually IgM is secreted, switching to IgG as the immune response progresses. In type I hypersensitivity, activation of CD4+ T helper cells (Th2) causes a switch to IgE production. IgE becomes bound to specific binding sites (IgE receptors) found on the surface of mast cells, and it causes the subsequent stimulation of these cells by exposure to the antigen that causes the anaphylactic response. An antigen that causes an allergic IgE mediated response is termed an allergen.

Mast cells are widely distributed throughout the body both in connective tissue (e.g. Under the skin), and in associated epithelial mucosa (e.g. Respiratory and intestinal epithelia). They contain prominent granules which contain a number of mediators of inflammation, notably histamine and leukotrienes; which are secreted after antigen binding to the cell surface IgE. The inflammatory mediators released by mast cells act principally on blood vessels and smooth muscle. In connective tissue, histamine causes dilation of vessels with increased blood flow to the surface and increased movement of fluid out of the blood stream (oedema). Around mucosae, the opposite effect occurs, causing constriction of airways and contraction of the smooth muscle in intestinal walls. The net effect depends on whether stimulation is local or systemic.

**Skin allergy** - stimulation of the connective tissue mast cells causes vasodilation leading to the red colour of the skin rash, and oedema leading to its raised appearance. This is often called urticaria, and is familiar as they type of rash caused by stinging nettles. The localised skin reaction is used in allergy testing, where small amounts of possible allergens and injected by pin-prick to see if they cause this kind of localised wheat-and-flare reaction. Substances known to be capable of causing skin-allergies include animal hair, natural latex proteins, certain chemicals and insect/plant stings.

**Hay fever and asthma** – If the allergen is inhaled the principal site of action will be the mucosal mast cells of the respiratory system. If this is principally in the nasal passages, the effect will be oedema in the nasal epithelial lining, and a consequent general irritation, as well as mucal secretion stimulation. It is the allergic reaction which causes hay fever (allergic rhinitis). If the allergen reaches the bronchioles of the lungs there will be contraction of the smooth muscle reducing the diameter of the airways as well as inflammation and increased mucus production. The result is asthma, with difficulty breathing in and (especially) breathing out. Substances known to be capable of causing hay fever and asthma include pollens, faeces of dust mites, animal hair proteins.

**Food allergies** – If the allergen is ingested, then it will act on at mucosal mast cells in the intestinal tract. The stimulation of the associated smooth muscle leads to vomiting and diarrhea typical of a food allergy. If the allergic substance can be absorbed and pass into the blood stream, it may also cause skin symptoms (rashes or itching). Substances known to illicit such responses include nuts, legumes (e.g soya), shellfish, milk, eggs and wheat.

**Anaphylaxis** – results from a systemic response to the allergen, causing immediate responses throughout the body. Though not common, it is potentially fatal and must be treated as a medical emergency (?code blue). Dilation of peripheral blood vessels causes symptoms of rashes and oedema, but more importantly it results in a dramatic drop in blood pressure which can affect organ function – known as distributive shock. Constriction of the bronchi causes difficulty in breathing. The patient will respond with an increase in respiratory and heart rates. There may also be symptoms of nausea, abdominal cramps and diarrhoea from the effects on the intestines.

Emergency Tx – aimed initially at hypotension relief, and then underlying inflammation. Lay down pt., raising legs to improve blood flow to trunk and head. An injection of adrenaline will act to constrict peripheral blood vessels and redirect blood to the organs. An intravenous drip may also be used to control b.p. Medications that maybe used include antihistamines and anti-inflammatory corticosteroids. If breathing problems persist then high-flow (100%) oxygen may be necessary and bronchodilators given by inhaler. Substances that may cause anaphylaxis in susceptible patients are those which pass freely around the body, including: Penicillin (esp. Previously by i.v.), local anaesthetics, X-ray contrast agents, bee/wasp sting venom and peanuts (the allergen can be absorbed fast enough to cause systemic effects).

Long term Tx is largely by avoiding known allergens. For those difficult to avoid like bees, or nuts, where traces maybe present in apparently innocent foods, patients may carry self-administered epi-pens (epinephrine auto-injectors) and a medi-alert bracelet I.C.E.