CRIPS Digest

Total biosynthesis of the tubulin-binding alkaloid colchicine

The alkaloid colchicine is a disrupter of tubulin polymerization and was approved for treatment of certain forms of arthritis and Mediterranean fever by the US FDA in 2009. However, the use of extracts of Gloriosa and Colchicum as anti-inflammatory agents has been widely reported in folklore. The activity of colchicine is presumed to reside in its tropolone ring system. Molecules carrying this unique ring system are microtubule toxins while structures where this ring system is absent are not as toxic. The substitution at the single nitrogen in the ring, especially its acetylation, is important for colchicine to retain its bioactivity. Nett and Sattely have utilized four enzymes involved in the biosynthetic pathway of colchicine in the rhizome of the flowering plant *Gloriosa superba* (Hindi: Agnishikha). The three identified enzymes were (i) CYP71DA12, which hydroxylates the methyl group of N-formyldemecolcine (m/z 400.1755) to form the hemiaminal (m/z 416.1704) which generates N-formyldeacetylcolchicine (m/z 386.1598) through the release of formaldehyde via a non-enzymatic route; (ii) alpha-beta hydrolase (ABH), which deformylates the hemiaminal intermediate to produce N-deacetylcolchicine (m/z 358.1649); and (iii) N-acetyltransferase (NAT1), which, in the presence of acetyl CoA, converts deacetylcolchicine to (-)colchicine (m/z 400.1755). These three enzymes were combined with other enzymes identified in an earlier work (doi 10.1038/s41586-020-2546-8) to reconstruct the biosynthetic pathway of colchicine in *Nicotiana benthamiana* (belonging to tobacco family). This bioengineered pathway had 17 enzymes from *G. superba* and 3 from other plants and gave rise to (-)colchicine which is the natural enantiomer. The final yield of the pharmaceutically active alkaloid in the heterologous system was 268 ng/g dry weight of plant. This work also shows that the common assumption regarding tissue-specific regulation of enzymes may need to be relooked at for production of secondary metabolites. (J. Am. Chem. Soc. (2021) 143: 19454-65)

Red- and far-red-emitting zinc probes with minimal phototoxicity for multiplexed recording of orchestrated insulin secretion

Zn^{2+} ion plays a critical role in several physiological processes like gene transcription and regulation of gene expression, biocatalysis, signal transduction and apoptosis. Hence, measurement of Zn^{2+} levels in a dynamic system becomes important. Fluorescence probes have formed an important bioanalytical tool for researchers working in this area. Techniques to measure the complete spectrum of Zn^{2+} ions, spanning over eight orders of magnitude, are lacking. High levels of Zn^{2+} (0.01-20 mM) are encountered in secretory granules and synaptic vesicles and photostable probes to measure this range are missing. Zhang et al. started with the classic red rhodamine-based probe, RhodZin-1. The hydrophilicity of the probe was improved by addition of morpholino auxochromes (confirmed by RP-HPLC) to form PKZnR-1. Unlike sulphonation, this strategy does not alter charge on the molecule. Increased hydrophilicity reduced the interaction of the probe with cell membrane. Both phototoxicity (green light illumination) and non-specific staining of cells were reduced with the addition of morpholino moiety. Since the range of concentration of Zn^{2+} to be detected is quite large (nM-mM), different groups with tunable affinity for Zn^{2+} were conjugated to RhodZin-1. While the main chelating group (N,N-diacetic acid) was retained, changes were introduced in the side chain. Substitution with methoxy, ethoxy, methoxyethoxy, o-aminophenol-N,N,O-triacetic and 2-pyridylmethyl groups gave rise to PKZnR-1, PKZnR-2, PKZnR-3, PKZnR-4 and PKZnR-5, respectively. Substitution of methoxy with the bulkier ethoxy group in PKZnR-2 reduced the affinity of the probe for Zn^{2+} (K_d increased from 28 to 33 µM). The modular method of assembling the molecules permitted facile synthesis of tunable probes. The synthesized probes exhibited high affinity (0.19-74 µM) as well as high selectivity (over other divalent ions like Ca^{2+}, Mg^{2+}, Fe^{2+}, Ni^{2+}, etc. and peptides like GSH and glucagon) for Zn^{2+}. The probe with the highest affinity for Zn^{2+}, viz. PKZnR-5, was able to detect insulin granule exocytosis in murine and human cell clusters. More importantly, cellular fusion events could be monitored over a longer time period as the probe exhibited negligible phototoxicity. Next, the authors attempted to increase the range of these probes to the far-red region. This will permit the simultaneous detection of Ca^{2+} along with Zn^{2+} in cells, a commonly encountered situation. Silicon-based probes have been widely used in this space. The conventional protocols employ harsh reaction conditions with low yields. In later studies, the chelator has been coupled to the fluorophore via an amide bond. The lone pair on chelator nitrogen is not able to quench the fluorophore efficiently, leading to faulty "turn-on" response. To overcome this, the authors developed a novel N-alkylation-based synthetic route introduced towards the end of the traditional route to give rise to the PKZnFR family. The overall yield was in milligrams. Fluorescence microscopes commonly available in the lab can be used to work with this probe as its spectral properties overlap with that of Cy5 (excitation and emission wavelengths of 654 and 676 nm, respectively). The fluorescence turn-on ratio of PKZnFRs ranged from 105-119 compared to 23 for PKZnR-1. These far-red Zn probes allowed the use of PKZnFR-3 (red, λex 647 nm) to record insulin secreted by β-cells in the presence of the mitochondrial stain, PK Mito Red (yellow, λex 561 nm) along with the nuclear stain Hoechst (blue, λex 405 nm) in islets isolated from transgenic mice expressing GCaMP6f (green, λex 488 nm). The authors hope that the designed probes and their orthogonal use will meet the needs of "4D physiology in big data era". (Angew. Chem. Int. Ed. Engl. (2021) 60: 25846-55)

Impact of simulated intestinal fluids on dissolution, solution chemistry, and membrane transport of amorphous multidrug formulations

As per the definition provided by Central Drugs Standard Control Organization (CDSCO), "Fixed Dose Combinations refer to products containing one or more active ingredients used for a particular indication(s)." Fixed dose combinations (FDCs) have found enormous importance in clinical practice, especially in the treatment of chronic diseases. FDCs improve patient compliance and reduce the economic burden on the healthcare system. However, the effect of different drugs on properties of each other, e.g. miscibility, solubility, active concentration, extent of ionization, etc., has not been investigated in a systematic manner. The use of amorphous solid dispersions may be adopted to improve solubility of poorly water-soluble drugs. For example, supersaturation/solubility of components of amorphous FDCs in a biologically relevant medium has not been studied rigorously. El Sayed et al. studied two combinations, the first one for anti-retroviral therapy, which combines atazanavir and ritonavir, and the second one for anti-hypertension therapy, which combines felodipine and indapamide. The crystallinity of all four drugs was confirmed by DSC and PXRD. The resulting two-drug formulations were amorphous. Conversion of amorphous form was seen to increase solubility in buffer and fasted state simulated intestinal fluid (FaSSIF). In a mixture of atazanavir and ritonavir using PVP as the matrix, the supersaturation of atazanavir and ritonavir in FaSSIF decreased by 50% and 30%, respectively, as compared to the single drug. Interestingly, in the second mixture, only marginal decrease in solubility was seen in case of felodipine in a mixture when compared with the free drug while indapamide showed significant decrease in dissolution at all time points of incubation when present in 1:1 ratio with felodipine. Mass-transport profiles showed similarity in transport pattern of drugs across membrane (regenerated cellulose) in either group, either when considered alone or in combination. No difference was observed in buffer or FaSSIF. Membrane transport was seen to decrease by ~40-50% for atazanavir and ritonavir when present in combination as compared to the drug alone. A similar reduction was seen in case of indapamide. However, no difference in flux was seen in case of felodipine, either alone or in combination. The flux ratio was ~1 in all cases suggesting that the complex media (FaSSIF) had no measurable effect on transport across membrane although the solubility ratio was tilted positively towards FaSSIF. This resulted in overestimation of the amount of drug actually being transported across the membrane and may account for the observed results. Amorphization led to the formation of a miscible system between uncharged atazanavir and ritonavir. Reduction in maximum achievable concentration in the multidrug combination was proportional to the mole fraction of each drug. The difference in dissolution of ritonavir component of the multi-drug formulation in buffer and FaSSIF was attributed to its differential solubility in the two solvents. The authors have proposed a formula by taking into account the amorphous solubility of the drug in buffer and the micellar contribution (difference in the observed solubility of amorphized drug in buffer and FaSSIF) to predict the solubility of the drug in a two-drug formulation. They project that the same formula can be extended to predict the solubility of individual drugs in multi-drug formulations in other surfactant-based biomimetic systems which exhibit solution behaviour similar to atazanavir/ritonavir. The equation could be used successfully to predict and experimentally confirm the dissolution behaviour of indapamide in a two-drug formulation with felodipine. The solubility behaviour of felodipine, however, could be correctly predicted only in the presence of lower concentrations of indapamide and not at higher concentrations. (Mol. Pharmaceutics (2021) 18: 4079-89)

Caffeine as a viscosity reducer for highly concentrated monoclonal antibody solutions

Due to their remarkable target specificity, monoclonal antibodies account for a major fraction of applications of biopharmaceuticals. High concentration, with resultant high viscosity, is a barrier to the use of monoclonal antibodies for manufacturing and therapeutic purposes. Intravenous infusion requires trained health practitioner which adds to the cost of therapy. Traditionally employed excipients like arginine may increase solution viscosity in a few cases while some other viscosity-lowering agents are yet to receive regulatory approval. Zeng et al. have investigated the use of the alkaloid, caffeine, which is acceptable to regulatory authorities, as a viscosity-lowering agent, with the aim of developing a sub-cutaneous formulation. The authors have compared the viscosities of two clinically important monoclonal antibodies, viz. infliximab (marketed as Remicade®) and ipilimumab (marketed as Yervoy®), by formulating them in 75 mM caffeine and in 100 mM NaCl and 100 mM arginine (ArgHCl). The procured antibodies were buffer exchanged and formulated in 20 mM phosphate-acetate buffer, pH 6.0 or 20 mM histidine buffer, pH 5.5, for infliximab and ipilimumab, respectively. Addition of NaCl or arginine led to no change or slight increase in viscosity of both antibody solutions. However, addition of caffeine led to ~77% reduction in viscosity of infliximab (150 mg/ml). A similar order of reduction was seen when ipilimumab (150 mg/ml) was reconstituted in phosphate-buffered saline (PBS). When formulated in 20 mM histidine buffer, pH 5.5, addition of caffeine led to reduction in viscosity of the antibody from 29 cP to 21 cP (28% reduction). Addition of caffeine lowered attractive protein-protein interaction in infliximab by 66%. No effect of caffeine was seen in case of ipilimumab. Biolayer interferometry showed weak binding between the two antibodies and caffeine. As Remicade® is sold as a lyophilized powder, the effect of caffeine on the lyophilized antibody was studied. Different concentrations of caffeine were used for lyophilization and no difference in reconstitution time was seen when compared with the control (no caffeine). Depending on the concentration of caffeine in the formulation, 60-80% reduction in viscosity of the reconstituted formulations was reported. The viscosity-lowering ability of caffeine was retained when the formulations were incubated at 4°C for 37 weeks. SEC-HPLC revealed no difference in the monomer composition in any formulation immediately upon reconstitution or after incubation for 12 weeks at 40°C between control and caffeine-containing formulation. Cation exchange chromatography too showed no difference in charge variants in the presence of caffeine. Yervoy® is marketed as a solution formulation. In the presence of caffeine, no significant difference in monomer content of ipilimumab (200 mg/ml) was seen as compared to control when stored at 4°C/16 weeks or 40°C/6 weeks. The degradation pattern of the antibody also remained unchanged upon storage. TNF-? neutralization assay and CTLA-4-based ELISA showed that caffeine had no effect on the functionalities of infliximab and ipilimumab, respectively, in vitro. As caffeine has multiple advantages in terms of regulatory acceptability and favourable pharmacokinetic profile, the use of caffeine as a viscosity-lowering agent in high dose formulations may be explored further. (J. Pharm. Anal. (2021) 110: 3594-604)

First COVID-19 DNA vaccine approved, others in hot pursuit

The Emergency Use Authorization of ZyCoV-D by the Drugs Controller General of India (DCGI) has been hailed as "a milestone for a nucleic acid technology". The DNA-based vaccine (encoding spike protein and carrying IgE secretion signal) against SARS CoV-2 has been developed by Zydus Cadila, an Indian pharmaceutical company. It uses a needle-free jet injector (PharmaJet® Tropis®) for intradermal delivery of the vaccine, which is classified as 'safe' by WHO. It has been proposed that an alternate delivery route may lower the cost of the vaccine. DNA vaccines offer several advantages like ease of processing, longer period of response and enhanced stability. The major concern in their use has been regarding the possibility to get integrated into genomic DNA although no evidence has been offered in the literature to this effect. The article highlights the different requirements and cellular targets of mRNA- and DNA-based vaccines. This is clear from the dose of ZyCoV-D which is reported to be 2 mg of plasmid DNA each for three doses as compared to two doses of 30 µg each for BNT162b2, an mRNA vaccine. Peer-reviewed clinical trial data for ZyCoV-D has not been made available yet. The article traces the history and early failures of DNA-based vaccines which paved the way for mRNA to be developed as a vaccine platform. The article notes that since ZyCoV-D is cloned in a commercially available plasmid and follows a generic manufacturing protocol, its production can be extended to third parties and hence availability of the vaccine will not be a factor of concern. The vaccine exhibited 67% inhibitory efficacy in case of symptomatic infection and was fully effective in averting moderate disease. These numbers, and their comparison with other approved vaccines, may not mirror their usefulness in a clinical setting as the emergence of new strains and post-hoc data analysis force the revision of available data with vaccines which have already been approved. (Nat. Biotechnol. (2021) 39: 1479-1485)