Advances in Preclinical Toxicology: Bridging In Vitro, In Vivo, and Translational Gaps

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ABSTRACT:
Even in the early days of medical progress, drug safety is at issue. In the past, it was fashionable to use animal testing, but it is expensive, ethically flawed, and it does not replicate human reactions. Researchers are therefore resorting to alternatives like in vitro (laboratory cell tests) and in silico (computer modelling).In vitro methods include the study of drug actions on cultured cells from humans to identify toxicity, whereas in silico methods rely on mathematical models, machine learning, and simulations to forecast a drug's behaviour and toxic profile.This review emphasizes new developments in the combination of in vitro and in silico toxicology strategies for the prediction of in vivo toxic effects in early drug discovery. It discusses major studies using these approaches, their limitations in extrapolating to humans, and opportunities of new technologies such as organ-on-chip systems and PBPK modelling. It emphasizes the growing importance of combining human-relevant laboratory models with advanced computational approaches to further anticipate safety, reduce animal usage, and accelerate the rate at which safer, better drugs appear in the clinic.

KEYWORDS: In vitro toxicology, In silico modelling, Preclinical drug development, PBPK modelling, Organ-on-chip, Translational toxicology.

INTRODUCTION:

The diversity and volume of chemicals are vast with over 204 million chemicals having been reported in the literature. (1,2) To guarantee the safety level of humans during chemical exposure over 100 million animals are utilized every year. (3) Experimentation using animals has raised ethical and cost-related questions among the scientific community. Subsequently, next-generation risk assessment (NGRA) has been brought in as a new strategy. (4) New strategies such as in silico and in vitro strategies have been developed to provide safety decision making without involving in vivo data. (5-8). Preclinical research encompasses in vivo animal investigations, toxicokinetics, and safety pharmacology. each step providing useful information to inform the next actions through ongoing feedback mechanisms. The integrated approach emphasizes the importance of predictive toxicology at every stage of development in reducing attrition rates through identification of potential safety issues at earlier development stages. (9)

Also, in vitro experiments cannot fully represent the dynamic interactions that a drug experiences in a living system. (10) Although in vivo experiments tend to be more indicative of clinical performance than in vitro tests, much difficulty remains in the precise translation of results from preclinical species to man. (11) Furthermore, in vivo experiments rely predominantly on animal experimentation, which not only is expensive but also poses significant ethical hurdles. In order to bypass these constraints and enable large-scale, clinically relevant screening for toxicity, in silico methods have been hailed as a possible saviour. But to be universally accepted, however, these computer methods have to be seriously thought through and rigorously tested to establish credibility in the scientific community(12).

Toxicology is changing by embracing new technologies such as high-throughput screenings, omics investigations, and mathematical modelling (13). It is moving from conventional techniques to contemporary approaches based on human biology and integrative multi-omics analysis (14). Advances in human-cell culture methodology allow in vitro models to replicate in vivo function over time, with an emphasis on knowing the mechanisms of adverse effects and biological processes. (15)

Organs-on-a-Chip: Conceptual Framework and Structural Features: Design concept:

In culture systems, both the external environment of the cells and their internal ecosystem must be controlled to allow for correct growth and function. (16) By combining micromachining technologies with cell biology, organ-on-a-chip systems have the ability to control external factors accurately and very closely simulate natural physiological conditions. (17) To correctly simulate physiological processes on the chip, dynamic mechanical forces, fluid shear stress, and concentration gradients must be included as well as appropriate cell patterning.

Fluid shear force:

Microfluidic systems enable continuous cell culture via perfusion with micro-pumps, which allow for a stable supply of nutrients and effective waste evacuation. This dynamic configuration better mimics in vivo conditions than static cultures. Additionally, fluid shear stress helps in the formation of organ polarity. (18) Notably, organ-on-a-chip systems impose necessary mechanical forces that affect the normal biological functions of endothelial cells.

(19) Organ-on-a-chip platforms can be controlled by a simple 'rocker' motion or a sophisticated, programmable pulsatile system, arranged in one loop to accommodate the needs of various organ configurations. (20)

Concentration gradient

On the microscale, fluid generally displays laminar flow, generating stable biochemical gradients that are to be accurately controlled over time and space. These flow-rate and geometry-controlled biochemical gradients are important in bioprocesses like angiogenesis, cell migration, and tissue invasion. Microfluidic devices simulate such complex physiological processes by controlling flow rates and channel geometries with the help of microvalves and micropumps, allowing for the creation of controlled, three-dimensional biochemical gradients. (21,22,23)

Pulsatile mechanical pressure:

Microfluidic devices permit the use of flexible, porous membranes that exert pulsatile mechanical forces. These types of mechanical cues have been seen as significant determinants of cell differentiation in physiologic processes. (24,25)

BRIDGING THE TRANSLATIONAL GAPS:

Three-dimensional (3D) cell culture models of the liver can be an appropriate platform for filling the gap between animal work and clinical studies for evaluation of accumulation and hepatotoxicity of nano biomaterials in the liver:

Nano biomaterials (NBMs) have been very promising in various areas of applications, such as medicine. Despite this, just as much as they have been promising, clinical application of NBMs has not been rapid. (26) This excessively high failure rate among nano biomaterials (NBMs) can be partly explained by the inadequacy of available preclinical screening techniques for determining their toxicity within the human body. Among the top reasons for nanomedicine product withdrawal from clinical markets is NBM-induced liver toxicity, which is commonly

associated with extensive accumulation in the liver. (27, 28) Like conventional molecular drugs, nano

biomaterials (NBMs) are subjected to a series of in vitro and in vivo preclinical tests prior to transition to human trials.

Usually, following preliminary screening tests, such as sterility tests, NBMs undergo a thorough analysis pipeline involving physicochemical characterization, in vitro analysis like haematology, cytotoxicity, and immunological assessment, followed by in vivo evaluation of pharmacokinetics, biodistribution, and tissue accumulation. (29)

With respect to the in vitro and in vivo evaluation, precise prediction of human-specific hepatotoxicity remains a major challenge for researchers. (29) Human liver is richly vascular, multitudinous organ, which is connected by the portal vein and hepatic artery and is supplied by the blood from the gastrointestinal tract and aorta, respectively. It is formed of a heterogeneous mixture of cells like the immune cells, biliary epithelial cells, fibroblasts, hepatic stellate cells, Kupffer cells, adult stem cells, and hepatocytes (the major functional cells). Furthermore, the cytochrome P450 (CYP450) enzyme family also plays a role in drug and nano biomaterial metabolism-modulated liver function. (30)

Zebrafish model:

zebrafish, a non-mammalian model that presents new, useful, and affordable ways of filling in the gap between in vitro and in vivo experiments. Zebrafish present several advantages compared to conventional in vivo models. They are inexpensive to look after relative to rodents, which is especially advantageous considering the high costs of NBM research. Interestingly, young zebrafish are not subject to ethical approval for use, according to the European Commission Directive 2010/63/EU, because they are unable to feed themselves. (31) Zebrafish share significant genetic and physiological similarities with humans, with approximately 76% of human genes having a zebrafish equivalent. This level of genetic similarity is akin to that found in mouse and chicken models, which have about 84% and 80% of human genes, respectively. (32)

Zebrafish model has been utilized in preclinical NBM research, including thioridazine-loaded PLGA particle evaluation by Vibe et al. (33), hydrophobic drug release from cyclodextrin- and dextran-based nanocarriers by Peng et al. (34), and mesoporous silica nanoparticle-mediated photothermal-controlled drug delivery by Yan et al. (35) There are few limitations in spite of merits.

Therapeutic and Toxicological Applications of Induced Pluripotent Stem Cell-Derived Cardiomyocytes in Drug Research:

Reliable test systems are needed to determine and characterize possible drug targets, screen compound libraries for therapeutic effects of interest, and determine the safety of potential drugs. Such systems can be established with primary cells, established cell lines, or animal models. In cardiovascular pharmacology, however, current cardiomyocyte-based test systems have several shortcomings that reduce their reliability and translational value.

It is not easy to obtain primary human cardiomyocytes since they are not easy to cultivate for long periods and they cannot be expanded in vitro. There are no immortalized human cardiomyocyte cell lines with good fidelity for the main characteristics of cardiac physiology, including action potentials. As a substitute, human cell cultures from embryonic origin, such as human embryonic kidney (HEK) cells, are frequently utilized to develop overexpression systems for studying prospective drug target molecules. (36) Although this method allows the examination of a drug's action on a given gene or molecular pathway, it fails to provide information concerning the overall effect of the compound on cardiomyocytes. Consequently, a lot of the existing research continues to depend on animal models. Transgenic mice, for example, are widely employed to investigate the physiological processes underlying human heart diseases. Species differences, however, prove to be a major obstacle when employing animal-derived cardiomyocytes to model human cardiovascular diseases. For instance, murine hearts contract around 6 to 10 times more frequently than human hearts and have briefer action potentials driven by various ion channels. Mutations of the KCNQ1 gene, which codes for the ion channel of the IKs current in human beings, may result in long-QT syndrome type 1 — a disease with abnormally long QT intervals on an electrocardiogram and a risk of potentially life-threatening arrhythmias. (37)

"Induced pluripotent stem cell (iPSC)-derived cardiomyocytes are of great promise to propel pharmacology and toxicology in three broad categories.". First, they offer opportunities for identifying new drug targets. Cardiomyocytes generated from patient-specific iPSC lines, especially from individuals with inherited cardiac conditions, can be used in vitro to uncover molecules involved in disease mechanisms that may serve as potential therapeutic targets. Second, these cells can be utilized in phenotypic screening assays to evaluate compound libraries for compounds that demonstrate beneficial cardiovascular effects.

Third, iPSC- derived cardiomyocytes play a valuable role in safety pharmacology. Assessing the potential cardiotoxic effects of drug candidates is essential, particularly their ability to prolong the QT interval or trigger life-threatening arrhythmias such as torsade's de pointes. (38)

Omics Technologies:

Omics is broadly targeted at the techniques for the simultaneous detection, characterization, and quantitation of sets of molecules in a single experiment. The technologies are selected depending on the type of biomolecule, i.e., MS for proteins, microarrays and NGS for nucleic acids, and NMR for metabolites. The genome dictates the overall blueprint of all living things, so small changes can have huge impacts at all scales of biology from cell to population. Furthermore, genotoxicity and mutagenesis are closely related with exceptionally strong association with tumorigenesis. (39)

Genomics and Epigenomics:

Promising prospects arise using sophisticated methods such as Genome-Wide Association Studies (GWAS), which mostly examine single-nucleotide polymorphisms (SNPs) to detect genetic variations between individuals of the same species or strain. The genetic differences are subsequently linked with measurable pathological characteristics, e.g., drug susceptibility or resistance, toxic substances, and other diseases, e.g., cancer. (40) Although initial genotoxicology was largely focused on assessing DNA damage produced by exposure to chemicals with direct or indirect genotoxicity—e.g., formation of DNA adducts or damage arising due to disturbed repair mechanisms—today there is growing interest in the contribution of genetic polymorphisms. Such polymorphisms have increased importance whether they occur due to fixed mutations or as markers of individual sensitivity to environmental stressors. But both strategies have lagged behind expectations in toxicology science, especially when it comes to chemical mixtures. The investigation of genetic differences in reaction to environmental exposures, such as chemicals, accelerated after the first human genome sequence was completed. (41)

Researchers saw a significant increase in the frequency of single-nucleotide polymorphisms (SNPs) in the nematode Caenorhabditis elegans after exposure to silver nanoparticles over several generations, in a recent study. Matsumura et al. also created a genome-wide approach to detection of mutations in Salmonella typhimurium strain TA100 with the purpose of detecting mutagenic signatures. This method used model DNA-alkylating agents like ethyl nitrosourea, methyl nitrosourea, and ethyl methane sulfonate (EMS) in order to determine their mutagenic activity. (42,43)

DNA methylation and post-translational histone modification, together known as the 'histone code,' are two of the best-studied epigenetic mechanisms. They are regulated by an array of enzymes, whose function can be changed or impaired by the administration of toxic substances. (44) Desaulniers et al. examined DNA methylation in the livers of pregnant rats treated with individual and combined organochlorine pesticides, methylmercury (MeHg), and polychlorinated biphenyls (PCBs). The study indicated a reduction in global levels of DNA methylation, alongside decreased expression in a critical methyltransferase enzyme. While these researches did not investigate upstream regulatory processes, they also underscore the need for investigation of epigenetic changes that have a significant impact on gene expression and regulation. Specifically, the findings underscore that exposure to mixture chemical exposures has the capability of significantly impacting epigenetics. (45)

Proteomics:

Proteomics entails the global analysis of the proteome, which includes the total complement of proteins and peptides involved in maintaining structure, regulating metabolism signalling, facilitating transport and defence functions, and controlling key biological functions like cell division, differentiation, apoptosis, and breakdown and recycling of biomolecules. In toxicological research, proteomics is employed to identify how chemical challenge modulates the proteome.

Relative to transcriptomics, proteomics generally makes fewer target detections and is therefore comparatively less cost-effective. Nevertheless, despite such limitations, its downstream location in the gene expression pipeline as well as its higher degree of independence from genomic annotations make proteomics an important tool in systems toxicology. (46)

Proteomics has also been applied to studies that have sought to evaluate potential human health effects. For instance, Hooven and Baird examined the actions of coal tar, diesel exhaust extracts, and certain individual polycyclic aromatic hydrocarbons (PAHs) against MCF-7 human breast cancer cells. Although common mechanisms were seen to exist, the study indicated that each PAH elicited a unique proteomic response profile. These findings indicate the complexity of extrapolating health risks from exposure to complex mixtures of pollutants because synergistic interactions can take place that cannot be seen when analyzing separate substances. Also, the zebrafish model, currently well known in biomedical science, provides additional useful information in this regard. (47)

The zebrafish model, now well-established as a valued surrogate in biomedical studies, remains to provide telling examples. For example, Yin et al. [61] treated wild-type (AB strain) young zebrafish, aged 30-90 days post-fertilization, with mixtures of diketone antibiotics to determine their biological effects. (48)

Metabolomics:

Metabolomics is a novel branch of analytical biochemistry and may be considered the endpoint of the "omics" cascade. While genomics is concerned with studying the entire genome to gain knowledge on the function of individual genes, most of the functional genomics analyses are today still founded upon the study of gene expression (transcriptomics) and global protein analysis (proteomics). (49)

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the principal analytical instruments in metabolomics. In mixture toxicology, studies have most commonly used NMR data, frequently employing aquatic organisms as model organisms. This follows from ecotoxicologists' pioneering application of 'omics' technologies to research complicated by the difficulty of investigating organisms with both uncharacterized physiology and largely unmapped genomes. Jordan et al., for instance, used NMR-based metabolomics to investigate how combinations of endocrine-disrupting chemicals (EDCs) such as bisphenol-A, di-(2-ethylhexyl)-phthalate, and nonylphenol, which are identified as novel environmental pollutants, influenced the liver and gonads of male goldfish (Carassius auratus). The fish were treated with environmentally relevant doses of these compounds, in the range of nanograms per liter, via water over 10 days. (50) Similarly, Xu et al. used gas chromatography–mass spectrometry (GC-MS)-based metabolomics to identify oxidative stress and energy metabolism disturbances in the livers of rats after insecticide chlorpyrifos and cadmium (Cd) exposure. (51) Xu et al. [66] used GC–MS metabolomics to study liver tissue in rats, indicating evidence of oxidative stress and disturbed energy metabolism after chlorpyrifos and cadmium exposure. (52)

Challenges and shortcomings of existing preclinical in vivo models in advancing cancer drug research:

In vitro experiments give basic knowledge of a drug's toxicity towards cancer cell lines, assist in determining interactions between the drug and the target, and enable identification of associated biochemical and gene expression pathways. (53)The principal aims of these animal models are to accurately simulate human disease

conditions and correctly evaluate the potential of new treatments. (54) Subcutaneous tumour xenograft models have been a longstanding first choice for the assessment of treatment efficacy and drug-target interactions, with end-points usually quantified by calliper measurements and by survival rate of the test animals. (55) The method has been responsible for the clinical efficacy of a number of therapeutic drugs, including trastuzumab in the treatment of HER2-overexpressing breast cancer, melphalan in

rhabdomyosarcoma, and vorinostat in cutaneous T-cell lymphoma. While it has given useful information regarding therapeutic responses, this approach comes with significant limitations, particularly for molecular-targeted treatments. The models tend to inadequately simulate the cellular heterogeneity and rich tumour microenvironment of human cancers. Despite significant advances in the development of tumour vasculature-targeting agents — such as vascular-disrupting and anti-angiogenic therapies — these nuances are not well reflected in conventional xenograft systems. (56,57) One important disadvantage of calliper-measured subcutaneous xenograft models is that they cannot provide an accurate measurement of metastatic disease. (58) As metastasis is responsible for most of the mortality associated with cancer, effective model systems for early-stage drug development against this condition are urgently required.

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS (PBPK):

Physiologically based pharmacokinetic (PBPK) modelling is a computational method used to model the absorption, distribution, metabolism, and excretion (ADME) of a chemical compound and its metabolites within the body. It uses mathematical equations to describe the intricate relationships between anatomical, physiological, biochemical, and physicochemical parameters. (59) PBPK models are of crucial importance in human health risk assessment, especially in applications such as dose-response analysis, exposure, IVIVE, and extrapolation of toxicity and dosimetry across species. In toxicology, various PBPK models have been developed for chemicals, with some being used to inform and assist chemical risk assessment. (60)
Machine learning methods have been utilized to forecast PBPK parameters from compounds' physicochemical properties in order to build PBPK models for numerous compounds with high efficiency. A list of representative recent PBPK studies conducted with machine learning methods is presented in an accompanying manuscript (61) Machine learning methods have the potential to assist with the building and updating of PBPK models. On the other hand, PBPK models can generate large simulated datasets that, when subjected to machine learning analysis, can discover important new insights.

A recent study by Cheng et al. (2020) reported a generalized PBPK model specific to nanoparticles in tumour-bearing mice. The model was constructed with the help of 376 datasets

spanning different types of nanoparticles. The model was then used to predict the tumour delivery efficiency of various nanoparticles based on four dose-dependent parameters, such as delivery efficiency at 24 hours, 168 hours, the sampling point at the end, and the peak delivery efficiency. (62)
Recent advances in artificial intelligence and machine learning have significantly promoted their use in pharmacometrics and toxicometrics. A notable development is the application of deep learning models grounded on neural ordinary differential equations (neural-ODE) that have been utilized to learn to construct pharmacokinetic models automatically from clinical data. (63) This deep learning neural-ODE method is expected to be used with PBPK models as well to further enable its applications in pharmacology and toxicology.

Ethical and 3R (Replacement, Reduction, Refinement) Issues:
Both the general public and scientific communities have long been concerned with the distress caused to animals during research. Being sentient creatures, animals should receive humane treatment and strict adherence to ethical standards. It was in response to this realization that William Russell and Rex Burch proposed a system in 1959 for reducing animal distress in scientific research. (64) And ever since, "applying humanitarian methods in animal research" has been described as the three R's principles: Replacement, Reduction, and Refinement. Nowadays, 3Rs principles are widely embedded in scientific research animals use laws and regulations across the globe. Directive 2010/63/EU is a case in point and is the European Union's law specifically aimed at protecting animals used in scientific research. (65)

Replacement:
Directive 2010/63/EU includes the following main clause: "An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available." Presently, Directive 2010/63/EU includes protection for all non-human vertebrates living beings, which entails independently fed larvae and mammalian foetuses in the last third of their normal development, as well as invertebrate groups such as cephalopods, such as octopuses and squids."

Replacement could be relative or absolute. (64) Absolute replacement involves experimental strategies where animals are absolutely not included throughout all stages of the research process. (66)

Cells/tissue cultures and biomaterials:
Cell and tissue culture in controlled conditions outside the living organism is generally regarded as one of the low-cost and easiest research alternatives to animal use. One modern solution for absolute replacement of animals in research is the use of biomaterials. These are biomaterials specifically tailored as entities that can act in isolation or within sophisticated systems for a wide range of purposes in research as well as in the clinic. The generation of various tissue structures has also been effectively illustrated through the application of 3D bioprinting technologies. (67,68)
3D bioprinting technologies employ tissue-specific, decellularized extracellular matrix components as bioinks to generate functional human tissue with closely mimicked natural physiology. The technique allows for the precise organization of cells and biomaterials to generate microstructures with defined biomechanical characteristics that mimic important properties of native tissue environments. These constructs accommodate interactions with the environment matrix, inducing tissue-specific functions, and are now useful tools for studying tissue maintenance and disease processes. (69)

Organoids culture:
Organoids are three-dimensional, self-organized structures derived from stem cells that capture the architecture, physiology, and functional properties of their respective organs. The small, in-laboratory replicas show tissue-specific functions and are able to simulate a number of different disease conditions, providing researchers with useful tools to explore organ development, pathology, and responses to therapy in a controlled in vitro setting. (70)

Organs-on-chips:
Organ chips are microfluidic cell culture systems that have been developed with very small channels which may be lined with different kinds of living cells to allow them to interact through their secreted factors and structural patterns. Organ chips develop a functional cellular network which replicates the physiological and pathological conditions of human organs both at tissue and organ levels. By mimicking critical biological functions and structures within a regulated in vitro setting, organ chips are sophisticated substitutes for animal models for the purpose of exploring disease processes, physiological processes, drug discovery, and toxicity testing. (71,72)

Reduction:
Where it is not possible to replace animals entirely in research, reduction must be pursued. Reduction means keeping the number of animals involved in experimental protocols at a bare minimum yet still capturing reliable and meaningful information. Under Directive 2010/63/EU, such a strategy involves promoting the design of programs permitting sharing of organs and tissues of animals euthanized and hence optimizing scientific use of the animals and reducing their need for more. New clinical therapies are usually developed through validation in preclinical animal studies, where heterogeneity of individual host responses is generally a problem. This problem is particularly evident in sepsis studies, an ongoing clinical issue. Under such animal models, septic challenges may induce far-reaching responses in subjects, which require more significant sample sizes to obtain valid statistical results. A practical solution to minimize the number of animals to be tested is through the reduction of experimental variability by utilizing exact, contemporary methods. Here, biotelemetry has demonstrated great potential in sepsis studies by monitoring physiological parameters in real time and hence enhancing the consistency of data and minimizing the number of animals utilized to derive relevant findings.

(73).

Refinement:

If every avenue in curtailing animal use in experiments is depleted and replacement is unachievable, refinement then becomes the priority. Refinement entails the alteration of procedures and care practices to minimize the pain, distress, or suffering caused to animals in research and enhance their welfare. As Russell and Burch initially explained, refinement consists of reducing, so far as is possible, any pain or distress caused to animals that remain within the bounds of scientific investigation.(64)

As Russell and Burch stated, a given research problem can be solved by a variety of different procedures, and an experienced researcher will always select "the quickest, most elegant, and simplest one." To promote the use of refinement techniques, Russell and Burch referred to the now-famous statement by Isaac Asimov: 'Violence is the last refuge of the incompetent,' and highlighted that causing unnecessary distress to animals is poor science and something to be avoided through more humane, reflective methods.

FDA's Predictive Toxicology Roadmap:

This six-part structure emphasizes the Agency's priorities and role in predictive toxicology, highlighting the importance of context of use and resolving toxicology issues related to FDA- regulated products. The FDA's Predictive Toxicology Roadmap also identifies areas where predictivity needs to be improved and points out emerging technologies that can meet these needs while being consistent with the principles of the 3Rs — Replacement, Reduction, and Refinement of animal use. (74)

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Conclusion:

The preclinical toxicology landscape is fast changing with the incorporation of highly developed in vitro, in vivo, and computational methods that seek to enhance the prediction of human drug and chemical response. Traditional models have delivered significant insights, but their inability to mimic sophisticated human biology has highlighted the requirement for systems that are more physiologically relevant. Technologies like organ-on-a-chip platforms, 3D bioprinting, and induced pluripotent stem cell-derived models are bridging the gap between clinical outcomes and experimental results. Moreover, the increasing use of machine learning, systems biology, and predictive toxicology tools further supports data interpretation and decision-making. As these technologies continue to evolve, they not only have the potential to improve the efficacy and precision of safety evaluations but also facilitate ethical research approaches through decreased use of animals. The future of preclinical toxicology is a harmonious, complementary system that brings the best of various approaches together to provide safer, more effective therapeutic agents for human health.

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