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Protein Therapeutics from Monolayer to Spheroids- A Model for Preclinical Investigations

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The arrival of recombinant insulin in the pharmaceutical market paved a new direction for the clinical potential of proteins. Thus, the pharmaceutical industry underwent a paradigm shift towards proteins as therapeutic moieties. A number of recombinant protein drugs are available and many more are in pipeline to target major diseases, such as cancer, viral diseases, cardiovascular diseases and endocrine diseases [1]. In many cases, the recombinant protein is effective and plays an irreplaceable role in the treatment of the pathological condition. Such as hemophilia, except blood coagulation factor, which has an extremely limited source, the treatment mostly relies on the recombinant coagulation factor [2]. Many recombinant proteins are in clinical practice for more than two decades with an optimal safety and absence of side effects. As in case of growth hormone (GH) therapy, where administration of the growth hormone in patients with severe GH deficiency and hypopituitarism was found to be safe for long- and short-term usage [3,4]. In terms of cancer protein therapeutics, currently only monoclonal antibodies, targeting over-expressed receptors, are prominently available for treatment of different tumors. The development of hybridoma technology for monoclonal antibody production revolutionized the treatment regime for solid tumors. Following approval of the first monoclonal antibody, rituximab, for clinical treatment of non-Hodgkin's lymphoma, a number of monoclonal antibodies have been generated and are at present in clinical use for treatment of solid tumors [5,6]. However, the clinical success and the long-term benefits of recombinant protein therapy for treatment of several diseases have fostered enormous interest in their application for treatment of cancer. Li and colleagues reported anti-tumor effect of novaferon, a novel recombinant protein produced by DNA shuffling of interferon-α [7]. Evaluation of novaferon bioactivity on tumor cells displayed reduction in proliferation of HepG2 cells in vitro and in vivo. This promising pre-clinical study has provided strong lead for clinical trial verification. Likewise, Zhang and colleagues demonstrated HER-2 receptor targeted activity of another recombinant protein e23sFv-Fdt-casp6 (immune-caspasse-6) in vitro and in vivo [8]. The recombinant protein e23sFv-Fdt-casp6 displayed inhibition of proliferation of HER-2 over-expressing A172 and U251MG in vitro, but not U-87 MG cells with undetectable HER-2 expression. Thus, the ability to produce targeted cytotoxicity makes the recombinant e23sFv-Fdt-casp6 a promising therapeutic alternative for Glioblastoma multiforme (GBM) treatment.

From the viewpoint of therapeutics, choosing the potential drug target is extremely crucial. In pathological conditions such as cancer, the major focus lies on taming the signaling pathways that have lost their inherent regulation or control, as aberrant signaling is the hallmark of this disease [9]. One such frequently de-regulated signaling pathway associated with pathogenesis is the AKT signaling pathway [10,11]. The AKT pathway is well documented to promote growth and survival, migration, angiogenesis and transcriptional regulation. The over-expression of pro-proliferation protein AKT and the dependency of the tumor for its proliferation make the AKT signaling pathway an emerging therapeutic target [10,11]. One of the reasons for amplified AKT function is the loss or down-regulation of PTEN function. PTEN, located on chromosome 10 is a dual specificity phosphatase, catalyzing de-phosphorylation of D3 position of phosphatidylinositol 3,4,5-trisphosphate (PIP3) to generate phosphatidylinositol 4,5-diphosphate (PIP2) thereby negatively regulating the AKT signaling pathway [12]. PTEN mutations have been reported to be

a major determinant that influence tumor development across tissues [13]. Missense, nonsense or frameshift mutations occur throughout the gene; however, hotspot mutations at specific amino acids have been identified as Arg130, Arg173 and Arg233 [14]. Although PTEN mutations have been associated with wide range of cancers, uterine cancer and glioblastoma multiforme have the highest percentages of PTEN mutations and homozygous loss [13]. Envisaging the crucial role of PTEN, one of the potential therapeutic strategies is replenishment of the PTEN function. Hence, we cloned, purified and characterized recombinant PTEN protein to determine its potential as an anti-proliferative therapeutic protein [15].

Following selection of the potential protein drug, the next judicious step is to choose an appropriate formulation for stabilization and delivery of the protein. Proteins possess a well-defined structure that provides it a specific function, thereby not interfering with other biological processes [16]. However, this defined structure is very delicate making it prone to degradation and disintegration. Further, some proteins function at intracellular locations making the appliance of a delivery vehicle indispensable. The field of nanomaterials has seen some remarkable advancements when it comes to assembling materials for delivery of protein, genes or drugs for therapeutic purposes, where one such notable class of nanomaterials is metallic nanoclusters [17,18]. Nanoclusters act as fluorescent probe providing an opportunity to track the moiety tagged with the nanomaterial, providing an edge over non-fluorescent class of biomaterials [19-21]. For our protein of interest PTEN, which is an intracellular protein, a delivery vehicle becomes imperative. Therefore, we formulated a novel PEGylated nanocomposite comprising of recombinant PTEN and silver nanoclusters. The silver nanoclusters synthesized using lysozyme as the template were spherical positively charged nanostructures of size 2 ± 0.5 nm. The binding of the PTEN with silver nanomaterials was followed by PEG coating to generate spherical nanocomposites of size 125 ± 10 nm. The uptake of the nanocomposites in monolayer culture of cancer cell line was determined by confocal microscopy based on the red luminescence of the nanoclusters in the nanocomposites. To assess the goal of PTEN protein therapy, we performed studies on monolayer culture of two different cancer cell lines, breast cancer MCF7 and gliobastoma U-87 MG. We demonstrated anti-proliferative and anti-invasive activity of PTEN on breast cancer cell line. In the multi-drug resistance U-87 MG we demonstrated successful combination therapy of the nanocomposites with drug erlotinib. Thus, this comprehensive study of the novel nanocomposite on cell lines provided a strong lead to the implication of PTEN in therapeutic applications [22]. However, a successful anti-proliferative performance in the cell culture based studies does not necessarily translate to an at par performance in clinical trials. One obvious reason is the lack of complexity in the monolayer culture system. This demands evaluation of the nanocomposite or any drug at another testing platform before having clinical relevance. It is to be mentioned that animal models have been routinely used in pre-clinical studies, however there is fundamental genetic, cellular and metabolic differences between human tumor and xenograft human tumors [23]. A detailed research suggests that there are notable differences in transformation of human cells and rodent cells. Human cells require more genetic alterations for oncogenic transformation as compared to their murine counterparts [24]. This does not reduce the importance of these studies, but suggests exploration of data from animal studies may require careful insights. A recent breakthrough in biological models was the upgradation from two-dimensional cell culture (monolayer) system to three-dimensional cell culture [25,26]. Three-dimensional (3D) culturing of cells takes into account the spatial organization of cells, the heterogeneity and the in vivo cell environment. With emerging research in this field, a plethora of protocols are available to generated spheroids, which can be scaffold free [27] or scaffold dependent [28]. Scaffold based 3D culturing helps to recreate an environment resembling in vivo organ structure and function thereby overcoming the limitations of monolayer cell culture. Synthetic hydrogels are the most widely accepted material for scaffold as they can mimic the biological environment of extracellular matrix [25]. Spheroids are self-assembling cluster of cells that represent the true depiction of morphological changes that take place during cell transformation [26]. These three dimensional structures can be directly created from a large number of cell types and provide excellent models for studying cellular signaling, cell migration, differentiation, survival, and growth [29]. Having successfully achieved anti-proliferative efficacy of our recombinant PTEN protein in monolayer cultures of cancer cell lines, we decided to take the research a step forward and evaluate the potential of our construct (nanocomposite, containing PEGylated nanoclusters and recombinant PTEN) on spheroid system. We demonstrated the generation of spheroids from monolayer cultures of MCF7 and U-87 MG cell lines. A facile and reproducible forced flotation method to generate compact single spheroid in each well was employed. This scaffold-free method was found to be very simple, robust and reliable for spheroid generation. The clustering of the cells to form the 3D structure (spheroids) was easily monitored by microscopy. The objective of performing a 3D cell culture analysis was to determine the efficiency of the nanocomposite on a complex system before it can be employed for animal studies or clinical investigations. Following internalization of the nanocomposites in the spheroid, investigation of spheroid viability, cell cycle analysis and gene expression revealed successful anti-

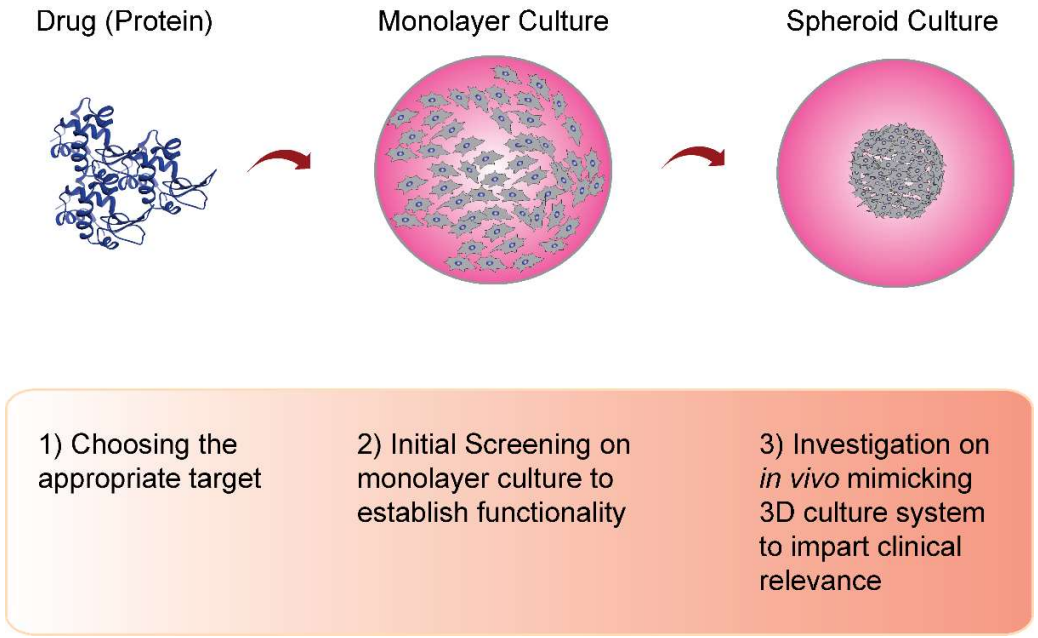

Figure 1: The schematic description of the drug testing platforms.

proliferative activity of the nanocomposites. However, along with testing the efficacy of the nanocomposite in the complex system, the study was also performed to highlight the differences between the monolayer culture and 3D culture systems. Just like the differences in the complexity of the two systems, there were differences in their response to the treatment regime. The complexity of the spheroid model was supported by higher doses of recombinant PTEN required to achieve similar effects as established in monolayer cultures. Thus, observation of both the monolayer cell culture and 3D cell culture systems lead us to draw two major conclusions. Firstly, although the two-dimensional monolayer culture does not entirely depict the in vivo microenvironment conditions, it is still very crucial for preliminary screening of any potential therapeutic protein in order to save time, labor and cost before exploring other platforms for further testing. Secondly, the three-dimensional model provides fruitful conclusion to therapeutic screening experiments. Hence, it is anticipated that additional modifications of the spheroid protocols in future may incur 3D model a valuable platform to establish pre-clinical investigation. The essence of the article is presented in Figure 1.

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