REPURPOSING OF BCS CLASS-II DRUG WITH CO-DELIVERY OF BIOACTIVE NANOCARRIERS AGAINST SKIN CANCER

Scope of application indicating anticipated product and processes:

The proposed work envisages repurposing and developing novel nanocarriers (nanosuspensions and nanoemulsions), encapsulating berberine, mangosteen and honokiol along with BCS Class –II drug for mitigation of skin cancer by activating p-53 dependent apoptosis and NF-kB down regulation activity. It is also anticipated that the obtained delivery system will provide the liberty in dose reduction of existing anticancer drugs and offer dosing compliance expected out of topical/oral delivery.

The delivery system will be fabricated contemplating the cost, stability, ease in scalability and most importantly, ensuring sustenance of anticancer drug within the cancerous cells which unfortunately ejects most of the bioactive via P-gp efflux mechanism and terminates as clinical failures.

Major Objective: (maximum 200 words)

The objective of proposed work includes:

- To Investigate the role of p-53 dependent apoptosis and NF-kB down regulation activity in skin cancer through co-delivery of BCS-Class-ii drug with bioactive nanocarriers and to analyze whether these bioactives nanocarriers succeeds in enhancing the effect of BCS-class-II drug by increasing solubility, bioavailability reducing toxicity and estimating the reduction in the release pattern of skin cancer biomarkers.
- To evaluate the histological and ultrastructural changes occurring as a result of co-delivery of co-delivery of BCS-Class-ii drug with bioactives in an established animal model of skin cancer and compare it with human cancerous skin.
- To develop topical/oral formulation of co-delivery of BCS-Class-ii drug with bioactive containing nanocarriers (nanosuspension and nanoemulsion) which utilizes cheaper excipients to solve the fundamental problems of skin cancer.

- To evaluate the usefulness of the combinational drug system where berberine, mangosteen and honokiol will reduce the complications associated with BCS Class-II drug in the disease and multipathway approach will help to treat skin cancer much efficiently.

Definition of the Problem: (maximum 200 words)

Skin cancer is a disease which is characterized as a process of abnormal cellular growth and proliferation. **Squamous cell carcinoma (SCC) is predominant in the Indian population rather than the Basal cell carcinoma (BCC**); **this is an epidemiological characteristic that sets us apart from the global trend.** The frequency of skin cancers in men and women is in the ratio of **2:1 i.e more in male than female**. Although, extensive studies have been carried out to combat the disease, yet, the most preferred treatment is the chemotherapy or surgical removal of the tumor. The existing conventional system has been failed to bring relief to the patients because they do not discriminate between cancerous cells and the normal replicating cells leading to increase in complications (eg:- hair loss, damage to gastro-intestinal epithelia etc.). Moreover, available treatment options of systemic and UV phototherapies have associated side effects like hepatotoxicity, nephrotoxicity, teratogenicity with oral retinoids and acitinic kerotosis with radiation therapy. Topical therapy is the mainstay of treatment for mild to moderate skin cancer and often the initial treatment for severe disease. About 80% of patients with skin cancer are treated topically. No single topical medication is perfect by itself in the management skin cancer. All these factors drive the need for safe and effective therapy for skin cancer with maximum patient compliance.

Novelty: (maximum 200 words)

Development of novel drug delivery devices encapsulating bioactives (using co-delivery of berberine, mangosteen and honokiol) along with synthetic drug for treatment of skin cancer for mitigation of skin cancer by activating p-53 dependent apoptosis and NF-kB down regulation activity involves multidisciplinary approaches scaling from molecular level of research to the development of cheaper delivery systems, by establishment of newer animal model of diseases and determination of the efficacy of these systems *in*

vitro and *in vivo*. Proposal exploits the vast amalgamation of novel delivery systems that can hinder solubility and bioavailability problems of BCS class-II drug, protect the bioactive drugs from the external environment such as photodegradation and pH changes, while reducing dose dumping by controlling the release profile. Moreover, developed nanocarriers at the site of action governs controlled targeting and reduced time of exposure at non-targeting tissues which increases the efficacy of treatments and reduce toxicity and side effects thus improving patient compliance and convenience.

Key words: skin cancer, p-53 dependent apoptosis, NF-kB down regulation activity, bioactives, immunosuppression, nanocarriers.

Introduction (under the following heads)

a. Origin of the proposal

Cutaneous malignant melanoma is the most aggressive form of skin cancer, with a high mortality rate. Various treatments for malignant melanoma are available, but due to the development of multi-drug resistance, current or emerging chemotherapies have a relatively low success rates. This emphasizes the importance of discovering new compounds that are both safe and effective against melanoma [1]. In 2015, about 90.5 million people were diagnosed with cancer [2]. About 14.1 million new cases occur each year (not including skin cancer other than melanoma) [3]. Consequently, it causes about 8.8 million (15.7%) human deaths [4]. Anti-cancer drugs including 5-fluorouracil (5-FU), cisplatin, etoposide, paclitaxel, and doxorubicin are commonly used to treat various cancers, such as cisplatin and doxorubicin in ovarian cancer, 5-FU in skin, colon and gastric cancer, paclitaxel and doxorubicin in breast cancer, and etoposide in small-cell lung cancer. However, these chemotherapeutic agents have evident side effects such as nausea, vomiting, loss of appetite, decreased immunity, oral ulcers, and other adverse effects [5-6]. In general, the anti-cancer drugs, such as cisplatin and doxorubicin favor abnormal triggering via programmed cell death (PCD) such as apoptosis, necrosis, necroptosis, and autophagy in normal cells as well as abolishing inflammation of damaged cells[7-9].

The main problems that exist with chemotherapeutic agents are severe adverse effects and multi-drug resistance formation. Some of the methods by which cancer cells become resistant to therapies are drug efflux systems, amplification of drug targets, or changes in drug kinetics [9–11]. Various strategies have been attempted to overcome drug resistance, such as the use of nanoparticles, liposomes and micellar drug delivery vehicles, with some reported successes [11]. The adverse effects of cancer chemotherapy can be treated symptomatically, but in some instances such secondary treatments may be very toxic, which is unacceptable to some cancer patients [12–15].

Itraconazole (ITZ), a common anti-fungal agent, has demonstrated potential anticancer activity, including reversing chemoresistance mediated by P-glycoprotein, modulating the signal transduction pathways of Hedgehog, mechanistic target of rapamycin and Wnt/β-catenin in cancer cells, inhibiting angiogenesis and lymphangiogenesis, and possibly interfering with cancer-stromal cell interactions. Clinical trials have suggested the clinical benefits of itraconazole monotherapy for prostate cancer and basal cell carcinoma, as well as the survival advantage of combination chemotherapy for relapsed non-small cell lung, ovarian, triple negative breast, pancreatic and biliary tract cancer. Preclinical and clinical data have proposed the use of itraconazole as a promising anticancer agent in monotherapy or in combination chemotherapy. A screen of US Food and Drug Administration (FDA)-approved drugs identified itraconazole as an anti-angiogenic agent in 2007 and as an inhibitor of Hedgehog signaling in 2010. With the aim of enhancing the therapeutic efficacy of itraconazole as anticancer drug, P-gp inhibitors were investigated in a clinical trial that reported unsatisfactory outcomes. In a recent study conducted on 29 patients with basal cell carcinoma (19 treated with itraconazole) , it was observed that the tumor area decreased by an average of 24% in 8 of the itraconazole-treated patients with accessible lesions. All these facts leads to conclude that the severity of cytotoxicity with these drugs remains as such in basal cell carcinoma. The most common side effects of ITZ are nausea, abdominal pain, and rash. Less commonly, gastrointestinal upsets have been reported, (including vomiting, flatulence, diarrhoea, and constipation), headache, dizziness, and peripheral neuropathy. Rare but serious side effects have included liver failure, chronic heart failure, and neutropenia. The evidence presented above suggests that ITZ has a number of anti-cancer

effects at clinically achievable doses, particularly in NSCLC, BCC, and prostate cancer. There is evidence that that it may also be applicable to a number of other cancers, including glioblastoma, breast, pancreatic, and ovarian. More generally, the main mechanisms of action investigated to date, antiangiogenic and Hedgehog inhibition, may serve to identify other cancer types where investigation with ITZ may be beneficial. We note that from research findings that with many targeted therapies suggest that resistance may become an issue with ITZ as a monotherapy, as it is, for example with another Hedgehog inhibitor, vismodegib. Hence, its co delivery with natural bioactives (berberine, mangosteen and honokiol) not only minimizes the side effects but also increases its therapeutic potential in lesser dose with additional skin scavenging effects.

Phytochemical compounds from extracts of plant roots, bulbs, barks, leaves, stems and others have shown promising potential as anti-cancer drugs, or for serving as lead compounds in the synthesis of new drugs. They are often utilized as traditional medicines in the form of home-made tinctures, teas, or crude extracts. Disadvantages of natural products and traditional medicines include variation in preparation methods and therefore also chemical composition, dosage determination and adjustment, and the suitable route of administration. Although much research on compounds of natural origin to produce new drug substances occurs, research, specifically aimed at naturally derived medicines to optimize dosages for the intended route of administration and to design the most effective dosage forms, has become essential[17-19].

Apoptotic cells have long been observed in cancers. For example, the high rate of apoptosis seen in basal cell carcinomas of the skin explained why these are relatively slow growing tumors, in spite of their high mitotic rate (19-23). Increased apoptosis was observed in irradiated tumors and those treated with cytotoxins, implying that treatments that increased the rate of apoptosis could be used to treat cancers (23).Similarly, Nuclear factor-κB (NF-κB) includes a family of signal-activated transcription factors which normally regulate responses to injury and infection, but which are aberrantly activated in many carcinomas. A wide variety of agents can activate NF-κB through canonical and noncanonical pathways.

It is known that p53 and NF-κB pathways play opposing roles in human cancer, with p53 acting as a tumor suppressor and NF-κB as cancer activator. although a number of studies have focused on identifying p53 activators and NF-κB inhibitors individually, few studies have investigated the molecules that target both the pathways simultaneously. Identifying molecules that simultaneously activate p53 and inhibit NF-κB would have great potential in combination therapy for cancer and various other diseases and could provide helpful tools to better understand the crosstalk between the p53 and NF-κB pathways. Therefore, one of the major objective and challenge of our research is to investigate p53 activators and NF-κB inhibitors from bioactive sources and develop them into suitable nanocarriers form depending upon their ability to target specific pathways or cells, thereby avoiding the risk of undesired side effects in skin cancer.

Objectives:

Multifunctional topical/oral nanosuspension and nanoemulsion can be formulated encapsulating berberine, mangosteen and honokiol along with BCS Class –II drug for mitigation of skin cancer by activating p-53 dependent apoptosis and NF-kB down regulation activity which is probably the biggest stymie of anticancer formulations. Furthermore, topical/oral delivery of BCS class-II drug has limitation owing to its poor bioavailability while the later gets extensively metabolized and fails to elicit its desired therapy. The hypothesis aims to negate these limitations by use of co-delivery of berberine, mangosteen and honokiol which could provide multifunctional targeting on skin cancer cell line. The approach is simple – inhibit, kill, destroy and prevent drug resistance development in inadvertently modifying skin cancer modalities. Oils act as sustained depot for drug release and all the three bioactives along with the BCS Class-II drug being lipohilic will have easy residence within the oil phase. The nanoemulsion as well nanosuspension formulation also offers protection to anticancer drug and bioactives that are usually light sensitive and degrades at room temperature. Furthermore topical/oral delivery has patient compliance as most anticancer formulations have parenteral approach due to toxicity and P-gp efflux hurdles. The hypothesis also aims to develop simple, easily scalable formulation devoid of complex

steps and exorbitantly priced excipients which renders the skin cancer therapy unapproachable in a developing economy like India.

Expected outcome:

It is expected that on completion of the studies proposed in the project, a robust multifunctional nanosuspension and nanoemulsion capable of carrying combination of synthetic anticancer drug (BCS Class-II drug) with natural bioactives (berberine, mangosteen and honokiol) shall be produced for the effective chemoimmunotherapy of skin cancer, even in cases of resistance and would restore the normal skin activity. The platform simultaneously would ensure that major side effects with existent therapy are also curtailed. The formulated multifunctional nanosuspension and nanoemulsion will be fabricated using biodegradable, biocompatible and inexpensive excipients with enhanced efficacy coupled with increased stability having potential to deliver drug to the cancer site circumventing all known barriers.

Review of status of Research and Development in the subject

International status

The incidence of both non-melanoma and melanoma skin cancers has been increasing over the past decades. Currently, between 2 and 3 million non-melanoma skin cancers and 132,000 melanoma skin cancers occur globally each year. One in every three cancers diagnosed is a skin cancer and, according to Skin Cancer Foundation Statistics, one in every five Americans will develop skin cancer in their lifetime. More than one-third of all cancers in the United States are NMSCs. Smaller surveys suggest that the incidence may have increased by as much as 65% since 1980. Despite efforts to address skin cancer risk factors, such as inadequate sun protection and intentional tanning behaviors, skin cancer rates, including rates of melanoma, have continued to increase in the United States and worldwide.

b. National status

In India, skin cancers constitute about 1-2% of all diagnosed cancers. Basal cell carcinoma is the commonest form of skin cancer worldwide, but various studies from India have consistently reported SCC as the most prevalent skin malignancy. BCCs have also arised as the progressive cutaneous tumors accounting for approximately 70% of all malignant diseases of the skin. The exact incidence in India is not known. But NMSC is known to be uncommon in Asians. Among dark-skinned individuals, SCC is commoner than BCC. Various studies from India consistently report SCC to be the most prevalent skin malignancy. In dark-skinned people, SCC often occurs in sites that have not been exposed to the sun and is often aggressive. Otherwise, exposure to sunlight is the principal cause of both BCC and SCC. The incidence of SCC increases more rapidly with age and with cumulative sun exposure than does the incidence of BCC. SCC in heavily pigmented skin often arises in association with scarring processes. The incidence of BCC appears to be increasing worldwide. Most of the nonmelanoma cancers were in the head and neck. The study contradicted some previous findings on rising skin cancers among younger people and concluded that NMSC incidence rates were leveling off. Although the presence of eumelanin in dark skin is protective against the development of NMSCs, cutaneous BCC and SCC are increasingly being diagnosed in the Indian population. This is possibly because of a myriad of chronic dermatoses that have been associated with malignant potential, particularly for SCC. Dermatologists should be aware of the predisposing lesions and follow them on long-term basis for early detection of malignant change. Histopathology is valuable in establishing the diagnosis and identification of the histologic subtypes of SCC and BCC. Well-differentiated SCC and nodular BCC represent the most frequent histopathologic variants reported from India.

c. **Importance of the proposed project in the context of current status**

Various studies have highlighted a paradoxically increasing trend of BCC with female predilection and higher percentage of pigmented lesions in Indians. This skin malignancy tends to be commoner in rural and agriculture based population. Major contributory risk factors include intermittent rather than constant UV exposure, cultural and lifestyle changes, cosmetic indifference, possible role of arsenic and pesticides, improved clinical awareness, and diagnostic facilities. The increasing cancer burden calls for the need of introduction of national screening program including mandatory annual skin examination by trained health professionals at the national level. Since early detection and treatment of lesions are crucial to decrease functional and cosmetic morbidity and costs, this study highlights the importance of improving awareness among general practitioners, public health workers, and general population. The clinical and epidemiological data collected during different studies thus thrive the need of the development of preventive and healing strategies to combat the disease in a cost effective manner.

d. Patent details (domestic and international)

International Patents

Sr. No.	Title	Patent No.	Inventee	Abstract
1.	Flavonoid compositions for the treatment of cancer	US 20170087125 A1 **Publication Date: Mar 30, 2017**	Daqing Wu	Compositions containing luteolin, quercetin, and kaempferol are provided. The compositions are useful killing cancer cells and treating cancer. Exemplary cancers that can be treated include, but are not limited to prostate cancer and head and neck cancer.
2.	Formulations including silver nanoparticles and methods of using the same	WO 2015057983 A1, **Publication date** Apr 23,	Ajay Pratap Singh, Sumit Arora, Seema Singh	A method for reducing or inhibiting damage to skin is disclosed herein. In some embodiments, the damage is ultraviolet (UV)

		2015		radiation-induced damage. In some embodiments, the method comprises applying to the skin prior to exposure to radiation damaging source, a formulation containing an effective amount of silver nanoparticles (AgNPs) A method for treating damaged skin, and formulations include AgNPs are also disclosed.
3.	Systems and methods for preventing cancer and treating skin lesions	US 8642655 B2, **Publication Date: 4 Feb 2014**	Benjamin Johnson	Systems and methods of using a composition containing at least one or more amino acids for topical application to skin to prevent cancer and treat skin lesions.
4.	Skin cancer prevention method and product	US 20100093674 A1, **Publication date Apr 15, 2010**	John R. Person	According to the present invention, the beneficial effects of UV radiation are obtained by incorporating vitamin D into the topical after-sun lotions and creams, applied to the skin after exposure to the sun to prevent its harmful effects. Application of the after-sun product provides vitamin D, which is activated by the skin to calcitriol for cancer prevention. Because calcitriol also promotes cellular growth and differentiation, the topical after-sun product with vitamin D may be of benefit for photoaging.
5.	Topical formulation for Use or in the	GB 2421183 A, date of **Publication**	Cutis Gerald	Topical compositions comprising a steroid selected from the group

	Treatment or Prevention of Skin cancer	**Jun 21, 2006**		consisting of ethisterone and derivatives thereof and trilostane and derivatives thereof and the use of these steroids in the manufacture of a medicament for the prevention or treatment of skin cancers that may be so treated by inhibiting angiogenesis and/or vasculogenesis. Such cancers include basal cell carcinomas, squamous cell carcinomas, melanomas and skin metastases.
6.	Topical formulation to treat skin disorders	US 20020031535 A1 **Publication date March 14, 2002**	Felix Sheffield	A topical formulation to treat skin conditions associated with skin cancer or other surface oriented skin disorders. The topical formulation includes the ingredients of copus or copper, bluestone, soda, turpentine, alcohol, and water. The topical formulation removes is applied to the affected area of skin to remove the associated skin disfiguremen

e. Specific Work plan

a. Methodology

Preformulation studies for the combinational drugs.

(a) Preformulation development

- Determination of solubility of combination of ITZ and bioactives (berberine, mangosteen and honokiol) in oils.

- Oil selection - Various GRAS approved edible oils and some semisynthetic oils will be selected for solubility of combinational drugs.
- FTIR, mass spectroscopy, NMR, DSC, partition co-efficient.
- Development of validated analytical method for simultaneous estimation of ITZ and bioactives (berberine, mangosteen and honokiol). All analytical parameters shall be scrutinized those that are specified in ICH (Q2) R1 guideline.

Preparation, optimization and characterization of nanoemulsion.

(a). Preparation of nanoemulsion

- The proposed nanoemulsion shall be prepared by high speed homogenization or spontaneous emulsification supplemented with probe sonication.

➢ Optimization of nanoemulsion

- Optimization will be carried out by varying lipids/or other component(s) of the formulation using QbD approach to get size below 100 nm.

Preparation, optimization and characterization of nanosuspension.

- The proposed nanosuspension shall be prepared by high speed homogenization supplemented with probe sonication.

➢ Optimization of nanoemulsion

- Optimization will be carried out by varying lipids/or other component(s) of the formulation using QbD approach to get size below 100 nm.

***In vitro* formulation characterization**

- The size and the surface charge (zeta potentials) –

  The size and zeta potential of the developed formulations shall be done using photon correlation spectroscopy and electrophoretic mobility, respectively on a Zetasizer. The sizes shall be measured in deionized water with a sample refractive index of 1.59 and a viscosity of 0.89.

- Shape and surface morphology –

  Shall be studied using transmission electron microscopy, scanning electron microscopy and atomic force microscopy. In order to perform transmission electron microscopy, appropriately diluted formulation shall be adsorbed onto a grid with carbon-coated form var film that is attached to a metal specimen grid. Excess sample shall be blotted off and the grid shall be covered with a small drop of staining solution (2% w/v uranyl acetate). It shall be left on the grid for

few minutes and excess solution shall be drained off. The grid shall be allowed to dry thoroughly in air and sample shall be examined in the transmission electron microscope.

- Encapsulation efficiency will be performed by employing a simultaneous validated reverse phase HPLC method specific for combinational drugs (bioactives with ITZ) being ferried. Procedurally nanoemulsions and nanosuspension will be centrifuged at in an ultracentrifuge to separate the aqueous phase (Sigma 3-18 K, SciQuip Ltd, UK) and oil phase. The oil phase will be dissolved in methanol and concentration of drug in the samples will be determined by the abovementioned HPLC method using suitable dilutions with mobile phase.
- Dissolution study will be carried out using dialysis membrane method. Drug containing nanoemulsions will be filled in hermitically sealed dialysis bags and immersed in a beaker containing phosphate-buffer (pH 7.4). The dissolution medium will be maintained in a stirred state in accordance with bio relevant conditions. Aliquots will be drawn at predetermined time intervals and fresh dissolution media will be added at each time point to maintain the sink conditions. The amount of drug released will be analyzed by reverse phase HPLC method as mentioned previously. Various release kinetic models like zero order, first order, Higuchi model and Korsemeyer–Peppas will be applied on in vitro release data to understand the drug release pattern from the developed formulations. Various kinds of stability studies including as prescribed by ICH and those specific to emulsions like freeze thaw, circulation, centrifugation and biological media stability will also be conducted.
- Scalability for batch nanoemulsion and nanosuspension - Scalability of the optimized nanoemulsion shall be performed to provide samples for efficacy and animal study. Furthermore, scalability shall also be performed for resolving technology transfer issues to gauge the factors which may affect the formulation from translating from bench to market.

***In vitro* cellular uptake and internalization visualization**

- Cell uptake study: Quantitative and qualitative estimation of cell uptake shall be determined for FITC loaded nanoemulsion and nanosuspension using cell-associated fluorescence assisted cell sorting (FACS Calibur, software cell quest Pro) and fluorescence microscope. Cancer cells will be seeded in six well plates and left overnight to adhere with plate surface. Subsequently, formulation will be incubated with cells followed by washing with PBS (pH 7.4).

Cell cycle distribution and apoptosis

- For cell cycle distribution study, flow cytometry will be performed using PI as a fluorescent marker. Annexin V and PI kit (Invitrogen, CA, USA) will be used to determine the mode and extent of apoptosis according to manufacturer's instructions. Cell associated fluorescence will be measured employing a flow cytometer to appraise the phase of cell cycle arrest.

Detection of apoptosis by acridine orange and propidium iodide

- The prepared nanoemulsion and nanosuspension induced death of A431 cancer cell line (skin carcinoma, human) was quantified using acridine orange and propidium iodide double staining. Briefly, treatment will be carried out in a 6-well microplate. The A431 cells will be plated at a concentration of 1×10^6 cells/ml and treated with nanoemulsion and nanosuspension at different concentrations (0, 2, 5,10, 25 µg/ml). The cells were incubated in 5% CO_2 atmosphere at 37 °C for 24 hr. The supernatant will be discarded and the cells will be washed twice using PBS. A 10 µl of fluorescent dyes containing acridine orange (10 µg/ml) and Propidium Iodide (10 µg/ml) will be added into the cellular pellet at equal volumes of each. The percentage of apoptotic cells will be determined in an improved Neubauer rhodium hemocytometer under fluorescent microscopy (Olympus CKX41).).

Development and characterization of topical and oral dosage form

The objective behind the fabrication of nanoemulsion and nanosuspension is to fabricate such a formulation which can fight against skin cancer synergistically i.e by reducing the release of biomarkers, decreasing inflammation and pain,restoring skin components through histopathology studies.

Following parameters form would be used for evaluation of oral dosage form:

- Pharmacokinetic profile of prepared dosage form
- Pharmaceutical factors like dissolution study, disintegration study, friability, etc

Following parameters form would be used for evaluation of topical dosage form:

- Drug Permeation/Retention study
- *Exvivo* Study using Confocal laser microscopy

- Skin Irritation Study

In vivo Pharmacological activity

The animal studies will be undertaken by the prior approval of IAEC of SRIP Kumhari .In this study, following biomarkers will be identified and evaluated on topical and oral application of the prepared formulations. They are:

- Apoptopic study
- Reactive Oxygen Species (ROS)
- Matrix metalloproteinase (MMP-1)
- Intracellular GSH Estimation
- NF-kB reduction study

In Vivo study by Ultraviolet radiation induced skin cancer model:

For Histological Studies

The skin tissues would be fixed in10 % chilled neutral formalin for 12 hr. at 4 º C. It will be Rinsed with distilled water, dehydrated alcohol in series, cleared in xylene and embed in paraffin wax to make tissue blocks. A Cut of 8 µm thick series section using rotary microtome will be done. Staining of the sections will be done using hematoxylin and eosin stains and photomicrographs will be taken.

***In vivo* pharmacokinetics and biodistribution studies.**

(a). **Tissue Distribution and plasma drug levels**

- Tumor bearing mice will be randomly arranged in two groups. These groups will respectively receive oral doses of free drug and developed multifunctional naosuspension dosage form at appropriate dose. Blood samples will be collected in heparinized tubes through retro-orbital route and animals will be sacrificed to harvest major organs (liver, heart, kidney, spleen) along with tumor at preset time points. A validated HPLC bioanalytical method will be used to determine the amount of drug in tissue and plasma.
- **Alternate strategies**: A validated LC-MS-MS bioanalytical method will be used to determine the amount of drug in tissue and plasma

b. Organization of work elements

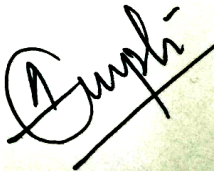
Period of study	Achievable targets
0-3 Months	1. Procurement of chemicals and necessary equipment. 2. Preformulation studies including assessment of purity of drug, bioactives, drug-excipients interaction and estimation of quantitative solubility of drug (ITZ+ bioactives). 2. Analytical method development (ITZ+ bioactives). 3. Validation of analytical method (ITZ+ bioactives).
3-16Months	1. Preparation of delivery systems incorporating combinational drugs (ITZ+ bioactives) in oral nanoemulsion and nanosuspension. 2. Optimization of process parameters. 3. *In vitro* formulation characterization. 4. Scalability of batch nanoemulsion and nanosuspensions (ITZ+ bioactives).
16-28Months	1. *In vitro* cytotoxicity assays 2. *In vitro* cellular uptake and internalization visualization 3. Cell cycle distribution and apoptosis 4. Detection of apoptosis by acridine orange and propidium iodide
28-32 Months	*Development and characterization of topical and oral dosage form* **Following parameters form would be used for evaluation of oral dosage form:** • Pharmacokinetic profile of prepared dosage form • Pharmaceutical factors like dissolution study, disintegration study, friability, etc **Following parameters form would be used for evaluation of topical dosage form:** • Drug Permeation/Retention study • Exvivo Study using Confocal laser microscopy • Skin Irritation Study

32-36 Months	***In vivo Pharmacological activity*** • Histological Studies (for topical dosage form) • *In vivo* pharmacokinetics and biodistribution studies (for oral dosage form)

Inter-institutional or industrial links, if any

The Institute has an inter-institutional link with:

- **University Institute of Pharmacy, Pt.Ravishankar Shukla University, Raipur, Chhattisgarh.**
- **Chhattisgarh Council of Science and technology(CCOST), Raipur, Chhattisgarh.**
- **National Institute of Technology (NIT), Raipur,Chhattisgarh.**
- **Arjuna Herbs and Extracts, Hyderabad, India**



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Date 27th Sept.2021