IN VITRO SCREENING TOOLS TO ACCELERATE NEW DRUG DISCOVERY: ALTERNATIVES TO ANIMAL TESTING

DABUR RESEARCH FOUNDATION

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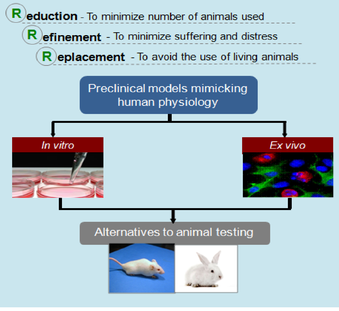
ABSTRACT

Screening of biological activities of new therapeutic compounds using *in vitro* assays provides a fast and reliable measure of efficacy and safety. These cell-based assays not only potentially accelerate the research of new therapeutic agents, but may also serve as alternatives to equivalent animal tests *in vivo* by reducing the number of animals and severity of procedures. At Dabur Research Foundation(DRF), we have developed a repertoire of *in vitro* models to assess activities of test agents in multiple therapeutic areas. Cytotoxic profile of anticancer compounds is evaluated in a panel of human and murine cancer cell lines with safety assessment in normal cell lines. Murine and human bone marrow cells derived Colony Forming Unit (CFU-GM) assays are employed to predict hematotoxic side-effects of anticancer drugs, which is European Commission for Validation of Alternative Methods (ECVAM) approved method to determine human MTD for neutropenia. Specialized cell-based screening models have been developed in the area of inflammation, such as Dendritic cells for systemic inflammation, keratinocytes/monocytes for psoriasis, lung/nasal epithelium cells for airway inflammation and allergy, fibroblasts for dermal inflammation and intestinal/colon epithelium cells for gastrointestinal-inflammation. Sophisticated *in vitro* models based on keratinocytes and sebocytes are available to screen anti-acne properties of cosmeticeutical/dermatological products. Skin health parameters such as anti-aging and anti-wrinkling potential are explored using skin-fibroblast cell lines. These *in vitro* assay systems contribute towards understanding of complex biological action of new compounds at cellular level. Shortlisting of hit/active compounds by *in-vitro* screening obviates the need of large number of testing animals while meeting 3R's principle of "Reduce, Refine and Replace".

INTRODUCTION

Animal models have been widely employed to assess pharmacological activity of new compounds. To predict toxicity, corrosivity, and other safety variables in addition to effectiveness of a new product for humans, traditional testing of new drugs involves the use of animals on a large scale. Registration, Evaluation and Authorization of Chemicals (REACH; regulation 1907/2006) is in effect in the European Union (EU) and safety information must now be provided on all chemicals that are either sold, manufactured or imported into the EU. Furthermore, an animal testing ban has been effective on chemicals to be used in cosmetics in EU from March 2009.

Realizing the importance in drug discovery, many alternative methods for animal testing are being developed, which have shown to lead to safer and more effective products for humans. Alternative testing methods have many advantages over traditional animal tests-including being more humane, reliable, accurate, more cost-effective, fast, practical and expedient. We have developed a huge spectrum of *in vitro*/*ex vivo* cell-based models in varied therapeutic applications such as cancer, hematotoxicity, immunomodulation, inflammation, dermatology and skin-care. These models profoundly increase the understanding of biological activities of test compounds and may serve as *in vitro* alternatives to similar tests conducted in animals.



IMMUNITY & INFLAMMATION

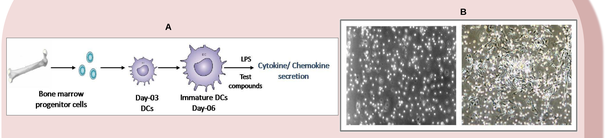


Figure 1 - Dendritic Cells (DC) assay (A) Generation of primary DC cultures from murine bone marrow (B) Morphology of day-3 and day-6 DCs

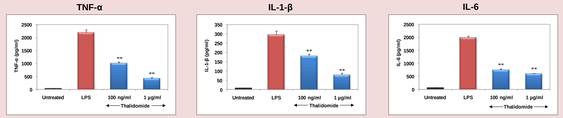


Figure 2 - Anti-inflammatory effect of Thalidomide in DCs based model

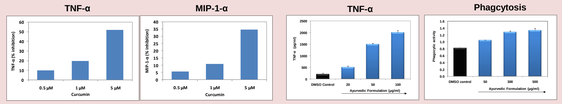


Figure 3 - Anti-inflammatory effect of Thalidomide in primary murine splenocytes

Figure 4 - Immunostimulatory effect of an ayurvedic formulation in DCs and macrophages (RAW264.7)

- Comprehensive range of *in vitro* & *ex vivo* models for identification of immunomodulatory and anti-inflammatory potential
- Cytokine and chemokine profiling in murine bone marrow derived DCs
- Phagocytosis in murine macrophages (RAW264.7)
- Anti-inflammatory effect by inhibition of LPS induced cytokine/chemokine in DCs and splenocytes

ANTICANCER ACTIVITY

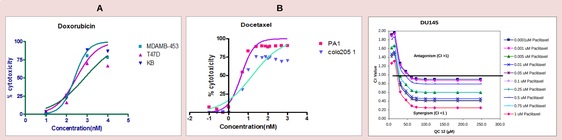


Figure 6 - Cytotoxic profile of (A) Doxorubicin and (B) Docetaxel in human cancer cell lines

Figure 7 - Synergistic cytotoxic profile of Paclitaxel and Quercetin in human prostate cancer cell line (DU145)

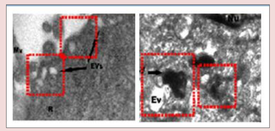


Figure 8 -Transmission electron micrographs of human breast cancer (MDA-MB-453) cells treated with nanoparticle formulation (60kx)

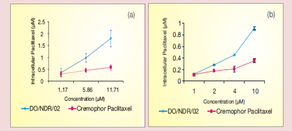


Figure 9 - Intracellular accumulation of Paclitaxel in (A) MDA-MB-453 and (B) PA1 (human ovarian cancer) cells treated with nanoparticle formulation

- Cytotoxicity screening in human and murine cancerous cell lines representing 15 – 20 different cancer types (ATCC/ECACC)
- Safety index in normal cell lines
- Synergistic/additive/antagonistic anticancer activity by combination index method
- Efficacy of targeted drugs
- Mechanism of drug uptake and subcellular distribution by Transmission electron Microscopy (TEM) and HPLC method
- Angiogenesis in endothelial cell based assay

DERMATOLOGY

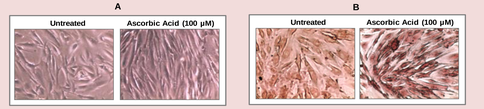


Figure 12 - Skin-health promoting effects of Ascorbic acid (100 µM) on (A) Morphology (B) Collagen synthesis in human fibroblast cell line (HFF-1)

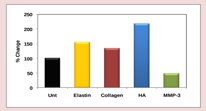


Figure 13 - Effect of Ascorbic acid on skin-health parameters in HFF-1 cells

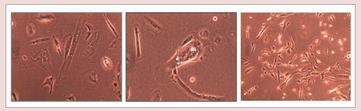


Figure 14 - Morphology of cultured human sebocytes

- *In vitro* screens to assess cosmeticeutical and dermatological products

No.	Indication	Model	End points
1	Skin-health	Human skin fibroblasts (HFF-1) and keratinocytes (HaCaT)	• Cell viability & proliferation • Collagen synthesis • Elastin, Hyaluronic acid, MMP-3
2	Anti-aging/ Anti-wrinkling	Human skin fibroblasts (HFF-1) and keratinocytes (HaCaT)	• Effect on skin health parameters in response to oxidative/UVB induced damage
3	Skin-lightening/ Vitiligo	Human melanoma cells (B16F10)	• Effect on tyrosinase activity
4	Dermatitis	Human skin fibroblasts (HFF-1) and keratinocytes (HaCaT)	• Effect on LPS induced cytokine/ chemokine secretion
5	Anti-acne	Human sebocytes and keratinocytes (HaCaT)	• Effect on LPS and *P.acnes* induced cytokine/chemokine secretion

RESPIRATORY INFLAMMATION/ALLERGY

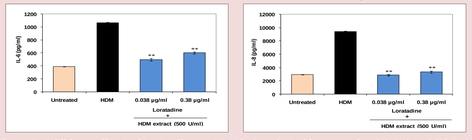


Figure 5 - Inhibitory effects of Loratadine on HDM-induced cytokine secretion (A) IL-6 and (B) IL-8 in human alveolar epithelium cell line (A-549)

- Downregulation of inflammatory markers in cell lines representing airway epithelium:

No.	Indication	Test system	Model	End points
1	Anti-allergic, Pulmonary inflammation, Asthma, Respiratory sensitization/ Irritation	• Human bronchial epithelial cells (BEAS-2B) • Human alveolar epithelial cells (A-549)	• Stimulation with inflammatory stimulus/allergen House Dust Mite (HDM) /Pollen grain extract / LPS	• Secretion of pro-inflammatory cytokines/ chemokines against stimulated levels
2	Rhinitis	Human nasal epithelial cell line (RPMI 2650)	• Stimulation with inflammatory stimulus/allergen House Dust Mite (HDM) /Pollen grain extract / LPS	• Effect on secretion of pro-inflammatory cytokines/chemokines against stimulated levels
3	Pulmonary inflammation/allergy	Histamine secreting Basophilic cell line (KU-812)	• Stimulation with Ionophore/ Compound 48/80	• Anti-histaminic effect
4	Pulmonary inflammation/allergy	IgE secreting cell line (U266)	• Stimulation with Ovalbumin	• Inhibition of IgE secretion

HEMATOTOXICITY

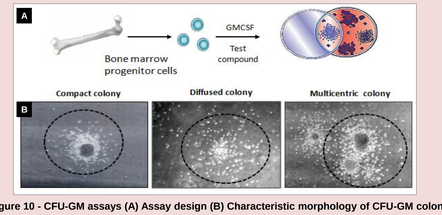


Figure 10 - CFU-GM assays (A) Assay design (B) Characteristic morphology of CFU-GM colonies

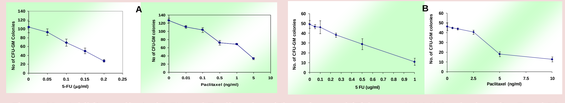


Figure 11 - Inhibitory effect of 5-FU and Paclitaxel on (A) murine and (B) human CFU-GM colonies after 7 and 14 days of continuous exposure respectively

- *In vitro* Colony Forming Unit (CFU) assays serve as predictive models for chemotherapy induced hematotoxicity

CFU in hematopoeisis	Growth factors	Type of prediction
CFU-GM (Granulocyte-macrophage)	GM-CSF	Neutropenia
CFU-E, BFU-E (Erythrocytes)	EPO, IL-3, SCF	Erythropenia
CFU-Meg (Platelets)	TPO, IL-3, SCF	Thrombocytopenia

- For anticancer drugs with neutropenia as dose-limiting toxicity, CFU-GM assay is approved by ECVAM Ispra, Italy (24th meeting, March 2006) to predict acute neutropenia in humans

$$\text{Predicted human MTD} = \text{Actual murine } LD_{10} \times \frac{\text{IC human CFU-GM assay}}{\text{IC murine CFU-GM assay}}$$

PSORIASIS

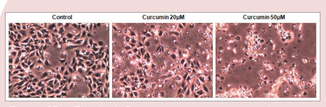


Figure 15 - Effect of Curcumin on morphology of human keratinocyte cell line (HaCaT) after 24 h

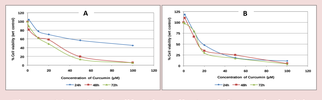


Figure 16 - Anti-proliferative effects of Curcumin in (A) HaCaT and (B) human monocytic cell line (THP-1) after 24, 48 and 72 h

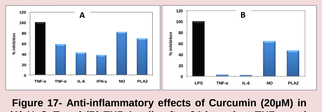


Figure 17- Anti-inflammatory effects of Curcumin (20µM) in (A) HaCaT and (B) THP-1 cells after 24 h against TNF-α and LPS stimulation respectively

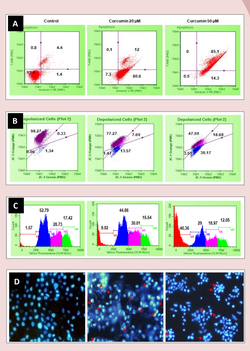


Figure 18– Induction of apoptosis by Curcumin in HaCaT cells (A) Annexin-V (B) Mitochondrial potential (C) Cell cycle (D) DNA fragmentation after 24 h

- HaCaT (human keratinocytes) and THP-1 (human monocytes) cells based model to evaluate anti-psoriatic potential
- Anti-proliferative effects – Time and dose kinetics
- Anti-inflammatory activity
  - Inhibition of cytokines in HaCaT cells against TNF-α stimulation
  - Inhibition of cytokines in THP-1 cells against LPS stimulation
- Pro-apoptotic effect
  - Increase in Annexin-V$^+$ cells, loss of mitochondrial potential, increase in sub(G0/G1) population and DNA fragmentation