

## RESEARCH COMMUNICATION

# Clinical Evaluation of 5-Fluorouracil from Transdermal Patches on EAC and DLA Cell-induced Tumors in Mice

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### Abstract

The aim of the present study was formulate and clinically evaluate 5-fluorouracil (5-FU) transdermal patches. Cytotoxicity was measured by exposing cell suspensions to increasing concentrations of drug from 10-100 µg/ml and performing viable cell counts by the trypan blue exclusion method. Results confirmed 100 µg/ml and 50 µg/ml of 5-FU to be cytotoxic to EAC and DLA cells. In mice, increase in the life span (ILS) by 87.1% with a maximum survival time of 30.5 ± 1.87 days was found with EAC cell-induced tumors, with an ILS of 88.1% and a maximum survival time of 39.5 ± 1.87 days for DLA cell-induced lesions with 5-FU transdermal patches. The results were statistically significant (p<0.01) compared to untreated controls. Pharmacokinetic studies in rabbits showed a t<sub>1/2</sub> of 29 ± 6 min, a C<sub>max</sub> (ng/ml) of 978.23, an AUC<sub>0-∞</sub> (ng/ml/h) of 1213.73 ± 14 and a T<sub>max</sub> (h) of 0.5. 5-FU from transdermal patches exhibited a half-life of 95 ± 0.5 min, a C<sub>max</sub> (ng/ml) of 863.25, an AUC<sub>0-∞</sub> (ng/ml/h) of 1567 ± 36 and a T<sub>max</sub> (h) of 1.5. Velcro protection jackets proved suitable in this study to stop mice licking, scratching and rubbing applied patches.

**Key Words:** 5-Fluorouracil - transdermal patch delivery - cytotoxicity - pharmacokinetics

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### Introduction

Delivering medicine to the general circulation through the skin is seen as a desirable alternative to oral application. Patients often forget to take their medicine, and even the most faithfully compliant get tired of swallowing pills, especially if they must take several each day. Additionally, bypassing the gastrointestinal (GI) tract would obviate the GI irritation that frequently occurs and avoid partial first-pass inactivation by the liver. Further, steady absorption of drugs over hours or days is usually preferable to the blood level spikes and troughs produced by oral dosage forms (Scheindlin, 2004). Transdermal drug delivery offers several important advantages over more traditional dosage forms. The steady permeation of drug across the skin allows for more consistent serum drug levels, often a goal of therapy. Intravenous infusion also achieves consistent plasma levels, but it is more invasive than transdermal drug delivery. The lack of peaks in plasma concentration can reduce the risk of side effects. In addition, if toxicity were to develop from a drug administered transdermally, the effects could be limited by removing the patch (Flynn, 1966).

5-Fluorouracil (5-FU) is an antimetabolite with promising antineoplastic activity against several premalignant and malignant conditions of the skin including Bowen's disease and superficial basal cell

carcinomas (Epstein, 1985, Bargman, 2003). 5-FU topical application has also been proven to be a valuable and safe treatment for actinic keratosis (Robins et al., 2002). 5-FU has been shown to be active against a variety of solid tumors, including those in breast, colon, rectum and cervix (Ansfield et al., 1962). After oral administration, 5-FU is poorly absorbed with significant variation in bioavailability ranging between 0 and 80%, following parenteral administration of 5-FU, there is a rapid elimination of the drug with an apparent terminal half-life of approximately 8-20 min (Diasio et al., 1989). In the present study we have developed transdermal patches of 5-FU and investigated for the cytotoxicity, maximum survival time (MST), increase in life span (ILS) for Ehrlich Ascites Carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cells induced solid tumor in mice and pharmacokinetic studies in rabbits.

### Materials and Methods

#### Animals

Swiss albino mice (6-8 weeks old) weighing 25-30gm were obtained from the Al-Ameen College of Pharmacy breeding section. The animals were kept in air-controlled rooms, fed with normal mouse feed (Sai Feeds, Bangalore, India) with water *ad libitum*. All animal experiments were performed according to the rules and conditions of the

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Institutional Animal Ethical Committee (IAEC) if the Government of India.

#### Chemicals

5-fluorouracil (5-FU) was purchased from Neon Pharmaceuticals (Mumbai), ethylcellulose (Ethocel average particle size  $9.7\mu\text{m}$ ) from Dow Chemical Company (Midland, MI) and PVP K30 from S.D. Fine Chemicals (Mumbai). Oleic acid, isopropyl myristate (IPM) and dibutyl phthalate were purchased from Rankem Chemicals (Mumbai). Backing membrane 3M CoTran 9720® (3M, USA), Release liner 3M Scotchpack 1022®, and Release Liner-Fluoro-polymer Coated Polyester film (3M, USA) were received as gift samples. All solvents were of analytical grade used without any further purification.

#### Cell lines

Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cells were obtained from Amala Cancer Research Centre, Thrissur, Kerala, India. The cells were maintained as ascites tumors in Swiss albino mice.

#### Preparation of 5-fluorouracil transdermal patches

A polymeric solution (10% w/v) was prepared by dissolving an ethylcellulose and PVP K-30 (ratio:8:2) formulation with 20mg of 5-FU, 40% v/w of di-butyl phthalate as a plasticizer 0.5% v/w of oleic acid, and 2% v/w of isopropyl myristate as a permeation enhancer (based on total polymer weight) in methanol as a solvent. The solution was poured into 6.06-cm diameter glass rings. The solvent was allowed to evaporate under ambient condition (temperature,  $32^{\circ}\text{C}$  and relative humidity, RH, 45%) for 24 h (the solvent was completely evaporated in 24 h). 3M CoTran 9720® was used as a backing membrane and 3M Scotchpack 1022® film as a release liner which could be removed before application of patch on the skin. The patches were cut with a circular metallic die of 3.4-cm internal diameter to give an area of  $10\text{ cm}^2$  and stored in airtight container under ambient conditions for several days prior to use. The drug was loaded into patches based on various pharmacokinetic parameters (Chandrashekar et al., 2008), such as volume of distribution (Vd), total body clearance (Cl<sub>t</sub>), and therapeutic plasma concentration (or) minimum effective concentration.  $12) \text{ The approximate dose per day} = \text{flux} (54\ \mu\text{g}/\text{cm}^2\ \text{hour} \times 24\ \text{hour} \times \text{surface area} (10\ \text{cm}^2) = 12.96\ \text{mg} \sim 13\ \text{mg}$ , desired flux = Clearance (180 ml/min) / minimum effective concentration (MEC) ( $0.05\ \mu\text{g}/\text{ml}$ ) / surface area ( $10\text{cm}^2$ ) =  $54\ \mu\text{g}/\text{cm}^2\ \text{h}$ . The drug concentration was not able to achieve the flux, so the concentration of drug was increased in the patch to 20 mg which yielded the desired flux (data not shown).

#### Design of Velcro protection jackets

The major problem encountered was how to protect the applied transdermal patch from being licked off, scratched off, and/or rubbed off during the experiments, once applied to the shaved dorsal surface of the skin of the mice. The Velcro jacket was designed, with small modifications according to the description of Su et

al., (1994). The Velcro jacket was made to cover the entire trunk of the mice and open at the top, which was designated for application of transdermal patch. The jacket protected the transdermal patch and allowed for good ventilation. It served its purpose quite well and the mice were able to function normally while wearing it.

#### Determination of in-vitro cytotoxicity activity of 5-fluorouracil in DLA and EAC cells

EAC and DLA cells ( $1 \times 10^6$  cells) were incubated with various concentrations of drug (10-100  $\mu\text{g}/\text{ml}$ ) in a final volume of 1 ml for 3 hours at  $37^{\circ}\text{C}$ . After incubation the viability of the cells was determined by the trypan blue dye exclusion method (Sheeja et al., 2004).

#### Determination of the effect of transdermal patch on survival time of DLA solid tumor bearing animals

DLA cells were aspirated, washed and suspended in phosphate buffer saline (PBS). Swiss albino mice were divided into three groups (10 mice/group). All the animals were induced solid tumor by injecting DLA cells  $1 \times 10^6$  cells/animal subcutaneously on the right hind limb. Group I was kept as untreated control, group III was administered with 5-fluorouracil through monolithic matrix transdermal patch applied on the dorsal surface of mice for 10 consecutive days. In group II drug was administered intravenously through tail vein for ten consecutive days. The death pattern of animals due to tumor burden was noted and the percentage of increase in life span was calculated using the formula  $T-C/C \times 100$ , where 'T' and 'C' represent the number of days the treated and control animals survived respectively.

#### Determination of the effect of transdermal patch on survival time of EAC-tumor bearing animals

EAC cells were aspirated, washed and suspended in phosphate buffer saline (PBS). Swiss albino mice were divided into three groups (10 mice/group). All the animals were induced tumor by injecting EAC cells  $1 \times 10^6$  cells/animal to peritoneal cavity. IV group was kept as untreated control, Group VI was administered with 5-fluorouracil through monolithic matrix transdermal patch applied on the dorsal surface of mice for 10 consecutive days. In group V drug was administered intravenously through tail vein for ten consecutive days. The death pattern of animals due to tumor burden was noted and the percentage of increase in life span was calculated using the formula  $T-C/C \times 100$ , where 'T' and 'C' represent the number of days the treated and control animals survived respectively.

#### Pharmacokinetic Studies in Animals

Experiments were carried out in healthy rabbits (New Zealand breed) with an average weight of  $2.58 \pm 0.14\ \text{kg}$ , they were divided into two groups, group-I (n=6) I.V route of administration of 5-fluorouracil (12 mg/kg) (and group-II (n=6) monolithic transdermal patch of 5-fluorouracil (20mg in  $10\text{cm}^2$  patch), animals were fasted for 24 h before the experiment, but allowed free access to water. During the experimental period, each rabbit was placed in a restraining stand. The marginal ear vein was cannulated using a Jelco catheter (Critikon, Chatanay-

Malabry, France) to collect blood. Blood samples (0.5 ml) was collected in heparinised 2ml eppendorf tubes before and over a period of experiment (0.0, 0.5, 1.0, 1.5, 2.0, 3, 4, 6, 8, 10, 12 and 24h). 5-FU in plasma was extracted (Barberi-Heyob et al., 1992), by procedure of liquid-liquid extraction using ethylacetate-methanol (95:5, v/v), drug concentration in plasma was measured using highly sensitive and validated Elisa reader (Biotek MQX200  $\mu$ Quant™, USA) in 384 well UV transparent clear plate (Life Sciences, Germany) at 266nm. The calibration curve was obtained by spiking drug-free plasma with varying amount of 5-FU (1000ng/100 $\mu$ L-10ng/100 $\mu$ L), good linear relationship was observed between the concentration of 5-FU with a high correlation coefficient  $r^2=0.993$ . The method was also validated for precision and accuracy. The drug-spiked plasma samples were prepared freshly on three different days and the resultant plasma was treated as per the procedure described. The intra-day variation was found to be less than 2.2%, thus, the results showed that this method was highly reproducible. Individual 5-FU time-concentration data sets were fitted with a non-compartmental non-linear regression analysis, employing with commercially available software WinNonLin 3.0Pro® (WNL, Pharsight Inc).

#### Histopathological studies

After 24 hours of administration of 5-FU through intravenous route and transdermal route, the animal was sacrificed, lungs and spleen was dissected and washed with cold saline, they were fixed in 10% neutral formalin and stained with hematoxylin and eosin stain. All the tissue samples were mounted on slides, examined and graded under light microscopy with 500 x magnification.

#### Statistical analysis

The results are expressed as the mean  $\pm$  SD. Statistical evaluation of the data was performed using Student's t test with Sigma stat.3.0, USA.

## Results

#### Determination of the in vitro cytotoxic concentration of 5-FU on DLA and EAC cells

In vitro cytotoxicity was observed by the exposure to 5-FU in different concentrations, to DLA and EAC cells. Reduced ability of DLA and EAC cells to survive, could be observed directly by examining microscopically using haemocytometer, stained by trypan blue. Cells were readily identifiable as a dark blue circles and the activity was estimated by counting the cells in field of view per 100 cells counted. Dose dependence was clear, with 24% cell death at 10  $\mu$ g/ml and 97% at 50  $\mu$ g/ml in the DLA case and 29% at 20  $\mu$ g/ml and 92% at 100  $\mu$ g/ml for EAC cells.

#### Determination of the effect of 5-FU through transdermal patch on the survival of DLA.EAC tumor bearing animals

The increase in life span (ILS%) was evaluated by comparing the mean survival by comparing the mean survival time of animals in each treated and untreated

**Table 1. Effect of Monolithic Matrix Transdermal Patch Delivery of 5-fluorouracil on the Life Span of Mice Bearing Tumors**

| Groups                           | Days survived (%ILS) |       |
|----------------------------------|----------------------|-------|
| Group-I (Control) DLA cells      | 21.0 $\pm$ 1.0       | -     |
| Group-II DLA +5-FU intravenous   | 24.0 $\pm$ 2.7       | 25.6  |
| Group-III DLA + 5-FU transdermal | 39.5 $\pm$ 1.9*      | 88.1* |
| Group-IV (Control) EAC cells     | 16.0 $\pm$ 1.0       | -     |
| Group- V EAC +5-FU intravenous   | 23.0 $\pm$ 2.7       | 43.6% |
| Group-VI EAC + 5-FU transdermal  | 30.5 $\pm$ 1.9*      | 87.1* |

ILS, Increase in life, \* $p < 0.01$  compared to the control case

control group. Both DLA and EAC cell induced tumor bearing mice treated with 5-FU in transdermal patch were found to have a significantly increased ILS (Table 1).

#### Pharmacokinetic study

After intravenous administration of 50mg/kg dose of 5-FU, the plasma concentration declined exponentially. The results confirmed that the distribution and elimination of 5-FU was very fast. The elimination half-life was only  $29 \pm 6$  min. The other pharmacokinetic parameters determined were  $C_{max}$  (ng/ml) 978.23,  $AUC_{0-\infty}$  (ng/ml/h)  $1213.73 \pm 14$  and  $T_{max}$  (h) 0.5. 5-FU from the transdermal patches showed half-life of  $95 \pm 0.5$  min,  $C_{max}$  (ng/ml) 863.25,  $AUC_{0-\infty}$  (ng/ml/h)  $1567 \pm 36$  and  $T_{max}$  (h) 1.5.

## Discussion

In vitro cytotoxicity was here observed to be dose dependent with both DLA and EAC cells, in line with earlier results using HeLa cells (McCarron et al., 1991). With  $10^{-2}$  M the cell growth was completely inhibited on exposure for 24 hours. It is well known that 5-FU is metabolized via two metabolic pathways; an anabolic pathway to fluoronucleotides including 5-fluorodeoxyuridine-5'-mono-phosphate, which produces the anticancer effects; and a catabolic pathway to fluorob-alanine (FBAL), which is excreted in the urine. A very high rate of 5-FU catabolism in the liver decreases the drug's anticancer effect. So it is believed that suppression of the liver catabolism could lead to an increase of anticancer activity. TDD bypasses the liver hepatic first-pass metabolism and anti-tumor activity has been shown to increase. To support TDD, a 5-FU derivative oral tegafur has been developed, which is catabolized very slowly in the liver compared to 5-FU (Masafumi et al., 1992). Tegafur was found to inhibit the growth of several transplanted solid tumor in animals (Kubto et al., 1978; Uchera et al., 1985), and it was effective against adenocarcinomas without causing severe side effects (Iigo et al., 1988). It is also reported that FBAL causes leukodystrophy of brain, which is the significant side effect of 5-FU administration (Koenig et al., 1978).

Therefore by administration of 5-FU through a non-invasive route, which by-passes the first pass metabolism in liver, therefore, a decrease FBAL is considered useful for diminishing this side effects. Woolfson et al., (1995b) investigated and reported that 5-FU bioadhesive patch can be used for local delivery to uterine cervix in the

condition of cervical intraepithelial neoplasia (CIN) which is common and potentially malignant, affecting women in a wide age group.

There was significant delay in the absorption of 5-FU through patches due to the controlled release of drug from the patches and half-life was statistically significant ( $p < 0.01$ ) compared to intravenous route in rabbits and such half-life is observed in humans in conventional route (Diasio et al., 1989). The cytotoxic concentration of the drug can be achieved through the TDD, effective drug concentration in therapeutic window can be maintained up to 24 h, with less toxicity and side effects.

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