

# Cytotoxicity, Anti-tumor Activity, Cumulative Skin Irritation and Sensitization Study of 5-Fluorouracil from a Transdermal Patch for Dalton's Lymphoma Ascites Cells

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Dalton's lymphoma ascites (DLA) cells were used as a model cell line to evaluate the cytotoxic concentration and anti-tumor activity of 5-fluorouracil (5-FU) through transdermal drug delivery (TDD) for solid tumors. Cytotoxicity was measured by exposing cell suspensions to increased concentrations of drug from 10–50 µg/ml and then the viable cells count was measured by the trypan blue exclusion method. The results confirmed that 50 µg/ml of 5-FU was cytotoxic to DLA cells. The tumor volume was 0.23 cm<sup>2</sup> and the increase in life span (ILS) was 81.8% with a maximum survival time of 39.5±1.87 days for 5-FU monolithic matrix transdermal patch in mice with solid tumors induced by DLA. The results were significantly different ( $p < 0.05$ ) compared to the untreated control, which had a maximum survival time of 19±1 days. The anti-tumor activity was very effective compared to intravenous therapy (ILS, 25.58%, maximum survival time, 24±2.7 days). No signs of erythema, vesiculations or bullous reaction were observed with the patches. The mean cumulative skin irritation and adherence scores for both mice and humans were zero and less than one, proving there was no irritation or sensitization. The patches adhered completely to the skin, without leaving any adhesive on skin (score=0 in human subjects). The transdermal patches had 100% flatness, a thickness of 150±0.03 mm, good content uniformity, folding endurance (> 500 foldings), elegance, smoothness, transparency, and flexibility. The Velcro protection jackets were suitable for the study and prevented the mice from licking, scratching, and rubbing the patches.

**Key words** — Dalton's lymphoma ascites cells, 5-fluorouracil, transdermal patch, cytotoxicity, anti-tumor activity

## INTRODUCTION

Transdermal drug delivery (TDD) systems are designed to deliver drugs through the skin to achieve systemic effects. Because they are designed for systemic use, topical application is expected to minimize side effects as much as possible. Initially, transdermal delivery systems were shown to be effective for the treatment of some systemic ailments, motion sickness and hypertension.<sup>1)</sup> Currently more than 35 TDD products are approved in the United states for the treatment of a wide variety of conditions including hypertension, angina, motion sickness, female menopause, male hypogonadism, severe pain, local pain control, nicotine dependence, and recently, contraception and urinary

incontinence.<sup>2)</sup> The advantages of TDD have been well-documented.<sup>3)</sup> They include therapeutic benefits such as sustained delivery of drugs to provide a steady plasma profile, particularly for drugs with short half-lives, and hence reduced systemic side effects, reducing the typical dosing schedule to once daily or even once weekly, hence generating the potential for improved patient compliance, and avoidance of the first-pass metabolism effect for drugs with poor oral bioavailability. Additionally, TDD represents a convenient, patient-friendly option for drug delivery with potential for flexibility, easily allowing dose changes according to patient needs and the capacity for self-regulation of dosing by the patient. Alternatively, TDD can be used in situations requiring minimal patient cooperation, that is, in situations involving administration of drugs by someone other than the patient. The non-invasive character of TDD makes it accessible to a wide range of patient populations and a highly acceptable option for drug dosing. 5-Fluorouracil (5-FU) is an

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antimetabolite with promising antineoplastic activity against several premalignant and malignant conditions of the skin including Bowen's disease and superficial basal cell carcinomas.<sup>4,5)</sup> Its topical application has also been proven to be a valuable and safe treatment for psoriasis and actinic keratosis.<sup>6,7)</sup> 5-FU has been shown to be active against a variety of solid tumors, including those in breast, colon, rectum and cervix.<sup>8,9)</sup> Apart from clinical usefulness for topical treatment of skin-related disorders, transdermal delivery of 5-FU may overcome certain limitations associated with oral and parenteral administration of 5-FU. After oral administration, 5-FU is poorly absorbed with significant variation in bioavailability, ranging between 0 and 80%, while 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.<sup>10)</sup> These two problems make 5-FU a suitable candidate for transdermal delivery. In the present study, we have investigated the cytotoxicity, anti-tumor activity, maximum survival time (MST), and increase in life span (%ILS) for solid tumors induced by Dalton's lymphoma ascites (DLA) cells. A Velcro protection jacket was designed for mice to protect the applied patch throughout the study.

## MATERIALS AND METHODS

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

**Animals**—Swiss albino mice (6–8 weeks old) weighing 25–30 gm 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 Institutional Animal Ethical Committee (IAEC) if the Government of India.

**Cell Lines**—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-FU Transdermal Patch

The polymeric solution (10% w/v) was prepared by dissolving an ethylcellulose and PVP K-30 (ratio:8:2) formulation with 20 mg 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°C and relative humidity, RH, 45%) for 24 hr (the solvent was completely evaporated in 24 hr). 3M CoTran 9720<sup>®</sup> was used as a backing membrane and 3M Scotch-pack 1022<sup>®</sup> 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 cm<sup>2</sup> 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,<sup>11)</sup> such as volume of distribution ( $V_d$ ), total body clearance ( $Cl_t$ ), and therapeutic plasma concentration (or) minimum effective concentration (MEC).<sup>12)</sup> The approximate dose per day = flux 54  $\mu\text{g}/(\text{cm}^2 \cdot \text{h}) \times 24 \text{ hr} \times \text{surface area } (10 \text{ cm}^2) = 12.96\text{--}13 \text{ mg}$ , desired flux = Clearance (180 ml/min  $\times$  MEC (0.05  $\mu\text{g}/\text{ml}$ )/surface area (10 cm<sup>2</sup>) = 54  $\mu\text{g}/(\text{cm}^2 \cdot \text{h})$ . 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).

**Thickness**—The thickness of the patch was measured using vernier calipers (Mitotoyo, Tokyo, Japan) at three different points of the film.

**Drug Content**—Drug-loaded polymeric films of 1 cm<sup>2</sup> were obtained from three different locations on the 10 cm<sup>2</sup> film, dissolved in 2 ml of methanol, sonicated for 10 minutes, and the volume was made up to 10 ml using 7.4 pH phosphate buffer. The absorbance was measured at 266 nm using a UV-visible spectrophotometer (UV 1700, Shimadzu, Tokyo, Japan).

**Folding Endurance**—The folding endurance value can be defined as 'the number of times a film can be folded at the same place without breaking.' This test is an index of the brittleness of the film; the lower the folding endurance value, the more brittle

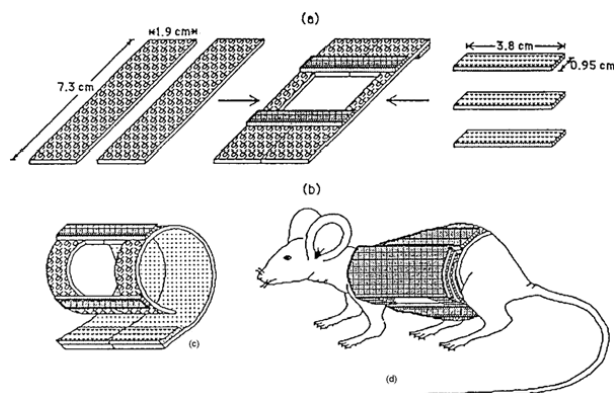
the film. It is an important test to assess the integrity of the film. The folding endurance was determined according to a previously described method.<sup>13)</sup> The films ( $2 \times 4 \text{ cm}^2$ ) were folded in the centre between the index finger and the thumb and then opened. This is termed 'one folding.' The procedure was repeated until the film showed breakage or cracks in the centre. The total number of folding operations was termed the 'folding endurance value.'

**Flatness** — An ideal transdermal patch should possess a smooth surface and should not constrict over time after application to the skin. Therefore, the flatness of the patches was studied by cutting them into strips and placing the strips on square glass molds ( $2 \times 4 \text{ cm}^2$ ) and then measuring their lengths. Percent flatness was determined as follows.  $\% \text{ Flatness} = L_1 - L_2/L_1 \times 100$ , where  $L_1$  and  $L_2$  are the initial length and final length of each strip, respectively.

**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.*<sup>14)</sup> 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. The details of the protection jacket are illustrated in Fig. 1. 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-FU in DLA Cells** — DLA cells ( $1 \times 10^6$  cells) were incubated with various concentrations of drug (10–100  $\mu\text{g/ml}$ ) in a final volume of 1 ml for 3 hr at  $37^\circ\text{C}$ . After incubation the viability of the cells was determined by the trypan blue dye exclusion method of Talwar.<sup>15)</sup>

**Determination of the Effect of Transdermal Patch of 5-FU on Solid Tumor Development in Animals** — Solid tumors were induced in 3 groups (10 mice/group) of mice by injecting DLA cells ( $1 \times 10^6$  cells/animal) subcutaneously into the right hind limbs. A 5-FU monolithic matrix transdermal patch was applied to the shaved (Whal hair clipper, model 9962, Whal Clipper Corporation, China) dorsal surface of group I mice [20 mg/dose/animal] daily for 10 days. Group II animals were administered 5-FU intravenously



**Fig. 1.** Schematic Illustration of a Velcro Jacket

(a) two pieces of Velcro together with opening, (b) joined pieces of Velcro, (c) the finished jacket wrapped around the trunk of shaved mice, and (d) once the patch was applied another piece of Velcro was covered over the opening to protect the transdermal patch.

(50 mg/kg body wt.) through the tail vein for 10 consecutive days. Group III served as an untreated control. The diameter of a developing tumor was calculated using the formula  $V = 4/3 \pi r_1^2 r_2$ , where  $V$  is the tumor volume, and  $r_1$  and  $r_2$  are the radii of the tumor in different planes. Readings were taken on every 5<sup>th</sup> day up to 30 days. The results were compared with the untreated control.

#### **Determination of the Effect of Transdermal Patch on Survival Time of 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). Solid tumors were induced in all the animals by injecting DLA cells ( $1 \times 10^6$  cells/animal) subcutaneously into the right hind limb. Group IV served as an untreated control. Group V was administered 5-FU through a monolithic matrix transdermal patch applied to the dorsal surface of the mice for 10 consecutive days. In group VI, drug was administered intravenously through the tail vein for ten consecutive days. Any deaths, including the cause, due to tumor burden were noted and the percentage 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 Cumulative Skin Irritation, Sensitization and Adherence Study in Animals**

Thirty animals were selected for the study. The study design was a controlled, repeat patch test, in other words, a monolithic matrix transdermal patch of 5-FU was applied to a shaved (Whal hair clipper, model 9962, Whal Clipper Corpora-

tion, China) dorsal portion of the mice. Patch application: one patch was applied to each animal for 23 hr (plus or minus 1 hr) daily for 21 days to the same skin site. When removing a patch, the site was evaluated for reaction and then a new patch was applied. Application of a test patch was discontinued at a site if predefined serious reactions occurred at the site of repeated applications. Skin reactions and patch adherence were scored using an appropriate scale. Dermal reactions were scored on a scale that describes the amount of erythema, edema, and other features indicative of irritations, as follows; 0 = no evidence of irritation, 1 = minimal erythema, barely perceptible, 2 = definite erythema, readily visible; minimal edema or minimal papular response, 3 = erythema and papules, 4 = definite edema, 5 = erythema, edema, and papules, 6 = vesicular eruption, and 7 = strong reaction spreading beyond the test site. Patch adherence was evaluated at the end of the wear period, immediately prior to removal of the patch. Scores corresponded to the percentage of patch surface in contact with the skin according to a 5-point scale of 0–4, where 0 = patch adhered > 90% (completely on), 1 = patch adhered 75–90% (edges lifting or center raised), 2 = 50–75% (half off), 3 = patch adhered < 50% (just hanging on), and 4 = patch not present on skin.

#### **Determination of Cumulative Skin Irritation Study, Sensitization and Adherence in Human Subjects**

— All of the human experiments were conducted according to the rules and conditions of the Institutional Human Ethical Committee (IHEC) of the Government of India. Informed consent was obtained from volunteers, and the study was approved by the IHEC. Sample size: 30 subjects, exclusion criteria: Dermatologic disease that might interfere with evaluation of the test site reaction, duration of study: 22 days. Study design: A randomized, controlled, repeat patch test of placebo patches (transdermal patch without active drug substance). One of each patch to be tested was applied to each subject. Patches should be applied for 23 hr (plus or minus 1 hr) daily for 21 days to the same skin site. When removing a patch, the site should be evaluated for reaction and then a new patch applied. Application of a test patch was discontinued at a site if predefined serious reactions occurred at the site of repeated applications. Application at a different site was permitted. The patches were applied on a clean, dry area of the forearm in all subjects. All subjects were examined for signs and symptoms of skin irritation. The primary outcome was

the skin irritation score, as measured by erythema. Erythema was graded by a trained clinical evaluator using a daylight blue incandescent lamp, on a 5-point scale: 0 = none, 1 = slight, just perceptible, 2 = slight, with definitive margins, 3 = moderate, obliteration of margins, and 4 = severe, vivid, spreading well beyond margins. Scores of 3 or greater were of an intensity that would most likely have been perceived as noticeable by the patient. Secondary evaluation criteria were patch adherence, adhesive residue, local skin reaction, and sensitization scores. Patch adherence was evaluated at the end of the wear period, immediately prior to removal of the patch. Scores corresponded to the percentage of the patch surface in contact with the skin according to a 5-point scale: 0 = patch adhered > 90% (completely on), 1 = patch adhered 75–90% (edges lifting or center raised), 2 = 50–75% (half off), 3 = patch adhered < 50% (just hanging on), and 4 = patch not present on skin. Immediately following removal of a patch, the amount of adhesive remaining on the patch and at the patch site was examined and graded on a 4-point scale, where 0 = none, 1 = light, 2 = medium, and 3 = heavy. Sensitization was scored on a 4-point scale, where 0 = not sensitized (no significant clinical signs or symptoms), 1 = mild sensitization (erythema and light oedema), 2 = moderate sensitization (erythema with infiltration, raised, spreading beyond the borders, with or without vesiculation), 3 = strong sensitization (large vesiculobullous, vividly red infiltrated plaques).

**Statistical Analysis** — The results are expressed as the mean  $\pm$  S.D. Statistical evaluation of the data was performed using Student's *t*-test with Sigma stat.3.0, U.S.A.

## **RESULTS AND DISCUSSION**

#### **Determination of the Cytotoxic Concentration of 5-FU on DLA Cells**

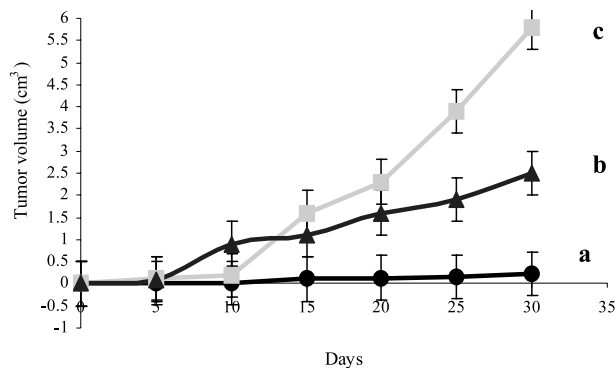
*In vitro* cytotoxicity was observed by exposure to 5-FU at different concentrations. Reduced ability of the DLA cells to survive, this could be observed directly by examining microscopically using a haemocytometer. Cells stained by trypan blue can be readily identifiable as dark blue circles and the activity can be quantified by counting the cells in the field of view per 100 cells counted. The results are shown in Table 1. Similar results were reported by McCarron *et al.*<sup>9)</sup> in HeLa cells with different concentrations of 5-FU ranging from

**Table 1.** Cytotoxicity of 5-FU in DLA Cells

| Concentration ( $\mu\text{g/ml}$ ) | Percentage of cytotoxicity (%) |
|------------------------------------|--------------------------------|
| 50                                 | 97                             |
| 40                                 | 78                             |
| 30                                 | 46                             |
| 20                                 | 31                             |
| 10                                 | 24                             |

DAL cells were incubated with different concentrations (10–50  $\mu\text{g/ml}$ ) of 5-FU and the percentage of dead cells was determined by the trypan blue dye-exclusion method.

$10^{-8}$  to  $10^{-4}$  M. The results have clearly shown that  $10^{-4}$  M is a cytotoxic concentration since cell growth was completely inhibited on exposure for 24 hr. It is well known that 5-FU is metabolized via two metabolic pathways; an anabolic pathway to fluoronucleotides including 5-fluorodeoxyuridine-5'-monophosphate, which produces the anticancer effects; and a catabolic pathway to fluoro- $\beta$ -alanine (FBAL), which is excreted in the urine. The very high rate of 5-FU catabolism in the liver decreases the drug's anticancer effect. Therefore, it is believed that suppression of the liver catabolism could lead to an increase in anticancer activity. TDD bypasses the liver hepatic first pass metabolism and may potentially increase the anti-tumor activity. To support the TDD, the 5-FU derivative oral tegafur has been developed, which is catabolized very slowly in the liver compared to 5-FU.<sup>16)</sup> Tegafur was found to inhibit the growth of several transplanted solid tumors in animals,<sup>17,18)</sup> and it was effective against adenocarcinomas without causing severe side effects.<sup>19)</sup> It is also reported that FBAL causes cerebral leukodystrophy, which is a significant side effect of 5-FU administration.<sup>20)</sup> Therefore, by administration of 5-FU through a non-invasive route, which bypasses the first pass metabolism in the liver, a decrease in FBAL is considered useful for diminishing this side effect. 5-FU has been shown to be active against a variety of solid tumors, including breast, colon, rectum and cervix.<sup>8,9)</sup> 5-FU is an ideal drug candidate for transdermal delivery into the systemic circulation that mimics the continuous IV route of administration. Woolfson *et al.*<sup>21)</sup> have reported that 5-FU bioadhesive patches can be used for local delivery to uterine cervix in cervical intraepithelial neoplasia (CIN), which is a common and potentially malignant condition affecting women of a wide range of ages.

**Fig. 2.** Anti-tumor Effect

(a) 5-FU monolithic matrix transdermal patch, (b) 5-FU intravenous injection, and (c) untreated control, against DLA induced solid tumors in mice. Drug was administered 24 hr after injection of DLA cells.

### Antitumor Activity of 5-FU through Transdermal Patch

There was a significant reduction in tumor volume in the animals treated with monolithic matrix 5-FU transdermal patch compared to the untreated control and intravenous therapy. Tumor volume in the control animals was  $5.8 \text{ cm}^3$  on the 30<sup>th</sup> day while 5-FU in transdermal patch was only  $0.23 \text{ cm}^3$  on the same day. The results are shown in Fig. 2. The data were significantly different ( $p < 0.005$ ) from the control.

### Determination of the Effect of 5-FU through Transdermal Patch on the Survival of DLA Tumor-bearing Animals

The increase in life span (%ILS) was evaluated by comparing the mean survival time of animals in the treated and untreated control groups. DLA cell tumor-bearing mice treated with 5-FU via a transdermal patch were found to have a significant increase in ILS. Control animals survived only  $21 \pm 1$  days after the tumor inoculation, while mice administered 5-FU intravenously and 5-FU patches survived  $24 \pm 2.7$  days and  $39.5 \pm 1.87$  days, respectively. Their ILS was 25.58% and 88.09%, respectively. The data are presented in a Kaplan-Meier graph in Fig. 3 and in Table 2.

### Physicochemical Parameter Evaluation of the Transdermal Patch

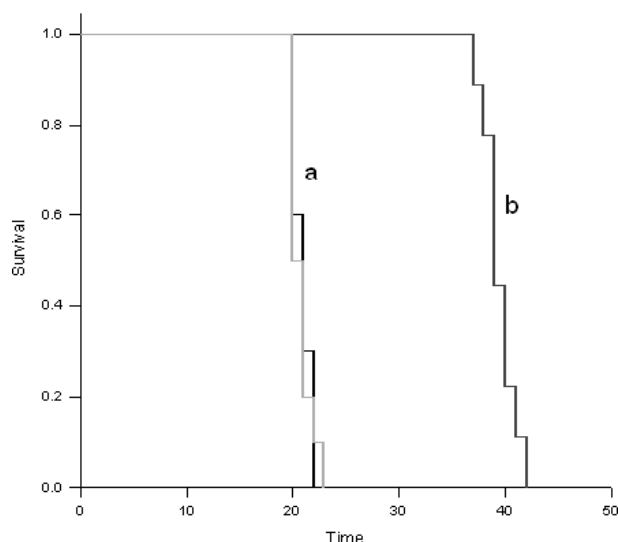
Patches were made with a drug loading of  $2 \text{ mg/cm}^2$ . Since we knew the influence of the amount of active ingredient loaded per square centimeter in determining the flux rate, we increased the drug loaded in the matrix (13 mg to  $20 \text{ mg/10 cm}^2$  patch) from which the MEC of 5-

**Table 2.** Effect of Monolithic Matrix Transdermal Patch of 5-FU on the Life Span of DLA Cell-induced Solid Tumors in Mice

| Groups  | No. of mice with tumors | No. of days survived | Increase in life (%ILS)<br>T-C/C × 100 |
|---|-------------------------|----------------------|--|
| Control   | 10/10                   | 21 ± 1               | —                                      |
| 5-FU intravenous, tail vein<br>(50 mg/kg body weight) | 10/10                   | 24 ± 2.7             | 25.58                                  |
| 5-FU monolithic matrix<br>transdermal patch           | 10/10                   | 29.5 ± 1.87*         | 88.09*                                 |

Values are ± S.D. ( $n = 10$ ), \* $p < 0.001$  (Student's  $t$ -test). Animals were treated with 10 doses of 5-FU intravenously or via a transdermal patch 24 hr after injection of DLA cells ( $10^6$  cells/ animal). Survival was observed for 45 days.

### Survival Analysis

**Fig. 3.** Kaplan-Meier Graph of Survival Rate of Animals with DLA Tumors by Administration 5-FU

(a) untreated control, and (b) monolithic matrix transdermal patch, Drug was administered 24 hr after injection of DLA cells.

FU flux can be achieved through the human skin (data not shown). The values were found to be  $2.0 \pm 0.1$  mg ( $n = 3$ ). The flatness study indicated 100% flatness for the patch and that it has no level of immediate constriction and that it can be suitably placed onto the skin surface where it will remain as such without constriction for a long period of time. One hundred percent flatness means that there is no constriction. The average thickness of the patches was  $150 \pm 0.32$  mm ( $n = 3$ ). The folding endurance test is an index of the brittleness of the film. The films tolerated more than 500 foldings ( $n = 3$ ), and were found to be more elegant, smooth, transparent and flexible, properties which can be attributed to the plasticizer. Each mouse wore a Velcro protection jacket during the entire experimental

period and the mice were housed individually. At the end of each dosing interval, the Velcro protection jacket was temporarily removed to enable treatment with finite dose formulations which were applied onto skin of the dorsal surface of the animal (*i.e.*, the treatment started the day after tumor inoculation and continued for ten consecutive days).

### Cumulative Skin Irritation Study, Sensitization and Adherence in Human Subjects and Animals

At each evaluation during the trial, the sites treated with patches exhibited less irritation, and at the end of the study the number of subjects with irritation (scores 1–4) was significantly less. Scores of 1–2 were observed among 2 human subjects. Moderate or severe erythema (scores of 3 and 4) was not observed in any human subject. The score in animals was 0, confirming there was no skin irritation or sensitization. Patch adhesion was greater, with 96.4% of humans and animals showing over 75% adhesion (scores 0 and 1). The percentage of patients with adhesive residue was significantly less with scores of zero. The overall percentages of subjects found to have no sensitization or local skin reactions such as pruritus, scaling and oedema. Patient compliance and effective patch adhesion are essential to the efficacy of TDD. The degree of irritation experienced by patients may be a factor in the degree of patient compliance with the treatment.

In conclusion, the present study has demonstrated that 5-FU could significantly reduce the development of DLA cell solid tumors and could also enhance the mean survival time of DLA cell tumor-bearing mice. Cytotoxic concentrations of the drug can be delivered through a TDD system. The non-invasive TDD route can decrease severe side effects such as cerebral leukodystrophy, and this could increase patient compliance. The Velcro protection

jacket served its purpose well and the mice were able to function normally while wearing this jacket.

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