8 Chapter 8: Combined *in-vivo* anticancer activity evaluation of optimized SD and TFG against melanoma

8.1 Background

In our previous studies (Chapter 6 & Chapter 7), the overall *in-vivo* tumor regression outcomes revealed the therapeutic potential of SD and TFG F2 for melanoma therapy either alone or as an adjuvant therapy with DTIC. Further, considering the fact that melanoma is the most aggressive and metastatic cancer, both the optimized formulation was administered simultaneously to provide local and systemic effects for the effective treatment of melanoma.

8.2 Objectives

- Development of syngenic transplantable tumor model in C57BL/6 mice
- Simultaneous administration of both the optimized formulations (SD & TFG F2) with DTIC and comparison with previously observed groups

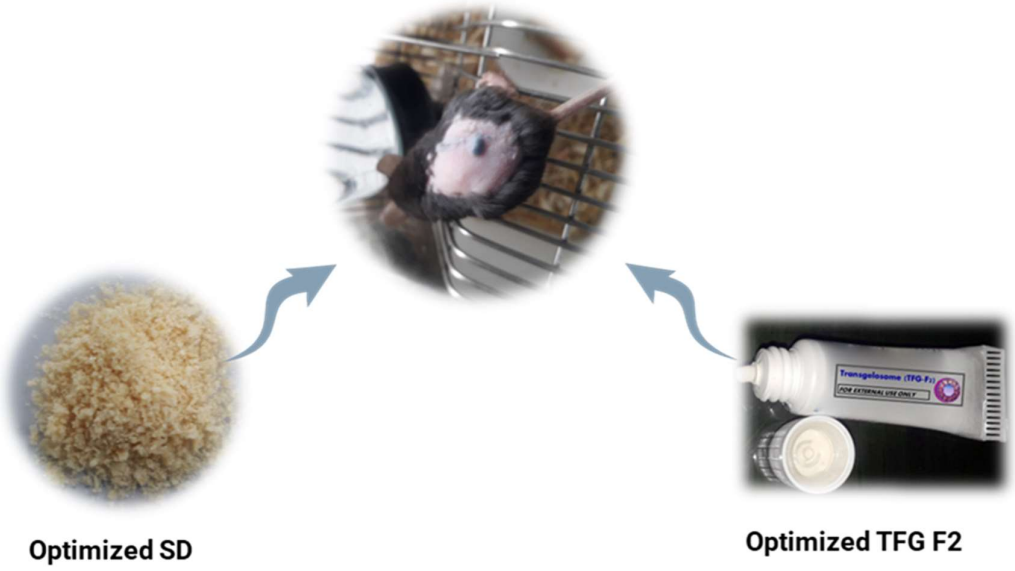

Figure 8. 1 Schematic representation of workflow of Chapter 8.

8.3 Methodology

8.3.1 Approval for animal experiments and standard experimental conditions

The guidelines of the CPCSEA were followed for care and experimentations on laboratory animals. Female C57BL/6 mice: 7-8 weeks old, 18 ± 2.013 g body weight were housed in cages with free access to standard food and water. All the animals were kept in a room (25 ± 1°C, 55 ± 5% RH) with 12 h of dark/light cycle and accustomed to the laboratory conditions over 1 week before experiments.

8.3.2 *In-vivo* anticancer activity of optimized SD and TFG in melanoma-bearing C57BL/6 mice

The *in-vivo* tumor induction was carried out as per the previously described method (Chapter 6). After 7 to 8 days, mice bearing palpable tumor (volume: 35 ± 3 mm^3) were treated with a combination of optimized SD and TFG F2, each of 100 mg/kg daily for up to 30 days. Also, the combination (optimized SD and TFG F2 each of 100 mg/kg daily b.wt) was administered daily with DTIC at a dose of 5 mg/kg i.p. every 2 days for up to 30 days [103]. The treatments were compared with the previous results of tumor control, optimized SD, optimized TFG F2, and DTIC groups.

8.3.2.1 Experimental design

A total of 20 adult female C57BL/6 mice were divided into 2 groups as follows and compared with previously studied groups (normal control, tumor control, DTIC group, optimized SD, and optimized TFG F2 group).

- **Group-I (optimized SD and TFG F2):** Tumor-bearing mice administered with optimized SD and TFG F2 each of 100 mg/kg daily b.wt. daily.
- **Group-II (DTIC + optimized SD + TFG F2):** Tumor-bearing mice administered with DTIC (5 mg/kg b.wt.) i.p. every 2 days, optimized SD and TFG F2 each of 100 mg/kg daily b.wt. daily.

8.3.2.2 Tumor regression analysis

The tumor regression analysis (TV, VDT, %TGI, tumor weight, and % ILS) and histopathology were carried out as per the described method under Chapter 6, section 6.3.5.9.1.

8.3.3 Statistical analysis

The mean and standard deviation (SD) for all experiments were calculated and expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test (for 3 groups or more) or students t-test (between two groups) at $p<0.05$ using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, California).

8.4 Results and Discussion

8.4.1 *In-vivo* anticancer activity of optimized SD and TFG in melanoma-bearing C57BL/6 mice

In-vivo tumor growth was observed through the changes in body weight during the study. The tumor control group showed an initial loss of body weight followed by faster weight gain than the treated groups and normal control group due to uncontrolled tumor growth (Figure 8. 2a). The body weight of treated groups (optimized SD+TFG F2 and DTIC+optimized SD+TFG F2) and the previously observed treated groups were found to be significantly lower ($p<0.05$) than that of the "tumor control group" due to the treatment effects.

The results of TV of various groups during the treatment period and at the end of the study were shown in Figure 8. 2b and Figure 8. 2c, respectively. A significant reduction ($p<0.05$) of the tumor volume was observed in the treated groups (optimized SD+TFG F2 and DTIC+optimized SD+TFG F2) compared to the tumor control group on the 30^{th} day of tumor induction. The calculated TV was in the order of "tumor

control group" > "SD group"> "SD+TFG F2 group" > "TFG F2 group" > "DTIC group" > "DTIC+SD+TFG F2" group (Figure 8. 2c).

The optimized SD significantly decreased ($p<0.05$) the TV compared to the tumor control group. The TFG F2 showed a significant reduction of TV ($p<0.05$) and comparatively higher anticancer activity than the optimized SD. The higher anticancer activity of TFG F2 is ascribed to the targeted site-specific delivery to the tumor microenvironment via transdermal application. The ultradeformable structure of the nanovesicular transferosome in the TFG F2 has the ability for deeper skin penetration and hence will reach the tumor cells present at the deeper region of skin. In contrast, the SD after oral administration leads to non-specific delivery to the tumor microenvironment of the skin and produces comparatively less anticancer activity. Hence, the combination of "SD+TFG F2" each at a dose of 100 mg/kg (total of 200 mg/kg) showed significantly less anticancer activity ($p<0.05$) than that of TFG F2 at a dose of 200 mg/kg. The DTIC alone showed significant reduction of the TV ($p<0.05$) compared to the tumor control group. Further, the "DTIC + optimized SD + TFG F2" group showed significant reduction of TV ($p<0.05$) compared to individually administered SD, TFG F2, and DTIC group. However, the "DTIC + optimized SD + TFG F2" group (5 mg/kg DTIC i.p.+ 100 mg/kg SD + 100 mg/kg TFG F2) showed slightly low anticancer activity compared to "DTIC + TFG F2" (5mg/kg DTIC i.p.+ 200 mg/kg TFG F2). This is due to the lower anticancer activity of SD compared to TFG F2.

The VDT (in days) of various treatment groups was found to be increased compared to the "tumor control" group due to the treatment effects (Figure 8. 2d). The VDT value in the case of the optimized "SD group" was found to be significantly higher

($p<0.05$) than that of the tumor-control group. The "TFG F2 group" showed a significant difference in the VDT compared to the "tumor control" group and a non-significant difference in the VDT compared to the SD group. The "DTIC group" demonstrated a significant improvement in the VDT ($p<0.05$) compared to the "tumor control." The "SD + TFG F2" group showed a non-significant difference in the VDT ($p<0.05$) compared to the SD group and TFG F2 group. A significant prolongation of the VDT ($p<0.05$) was noticed in the case of "DTIC + SD+ TFG F2 group" compared to the "tumor control group" and all treated groups. At a 95 % level of significance, all the treatment groups showed prolongation of VDT compared to the "tumor control" group. However, some non-significant differences in the VDT among various treatment groups are due to the uncontrolled rapid cell proliferation at the beginning of the tumor progression. During the study, it was observed that most of the tumors attained double of their initial volume within 8-10 days. The results of extracted tumor weight at the end of the study were represented in Figure 8. 2e. The tumor control group showed the highest tumor weight. In contrast, all the treatment groups showed a significantly lower ($p<0.05$) tumor weight. The optimized SD or TFG F2 or DTIC significantly decreased ($p<0.05$) the tumor weight compared to the "tumor control group." The "SD + TFG F2" group (each at a dose of 100 mg/kg) showed significantly lower ($p<0.05$) tumor weight compared to the optimized SD (200 mg/kg) group and significantly higher tumor weight ($p<0.05$) compared to the TFG F2 group (200 mg/kg). The result is in well accordance with the results of TV. "DTIC + SD+ TFG F2 group" showed a significant loss of tumor weight ($p<0.05$) compared to control and all treatment groups.

All the treatment groups showed a % TGI of more than 50%, and a value of >50% was considered to be meaningful [104]. The TFG F2-treated group showed a

significant improvement ($p<0.05$) of % TGI compared to the optimized SD-treated group. The "SD + TFG F2" group showed a significantly low % TGI ($p<0.05$) compared to TFG group and a non-significant difference with the SD-treated group. The "DTIC + SD + TFG F2" group demonstrated a significant improvement of % TGI compared to all treatment groups, representing the efficiency of adjuvant therapy. The photograph of representative tumors from each group at 30^{th} day was shown in Figure 8. 3a.

The life span of treated groups was found to increase compared to the "tumor control" group. The results of the percent survivorship of various groups were graphically represented as a Kaplan-Meier survival plot (Figure 8. 3b). The % ILS were SD: 84.4, TFG F2: 93.2, DTIC: 99.2, SD + TFG F2: 89.2, and DTIC + SD + TFG F2: 112.8 %. An increase in the life span by 25% or more over that of tumor control was considered as effective antitumor activity [104]. Overall, the *in-vivo* study revealed the stronger antitumor activity of TFG F2 compared to optimized SD. The improved efficacy of the standard anticancer drug (DTIC) was noticed during the use of SD or TFG F2 as adjuvant therapy. The TFG F2 was found to be a good adjuvant with DTIC than that of SD. Hence, a combination of DTIC with TFG F2 (5mg/kg of DTIC i.p. every 2 days + 200 mg/kg of TFG F2 daily) was found to be more effective than DTIC + SD + TFG F2 (5 mg/kg of DTIC i.p.+ 100 mg/kg of SD + 100 mg/kg of TFG F2). The transdermal application of standardized PLFEE-loaded TFG F2 showed a good adjuvant with the DTIC for the treatment of melanoma.

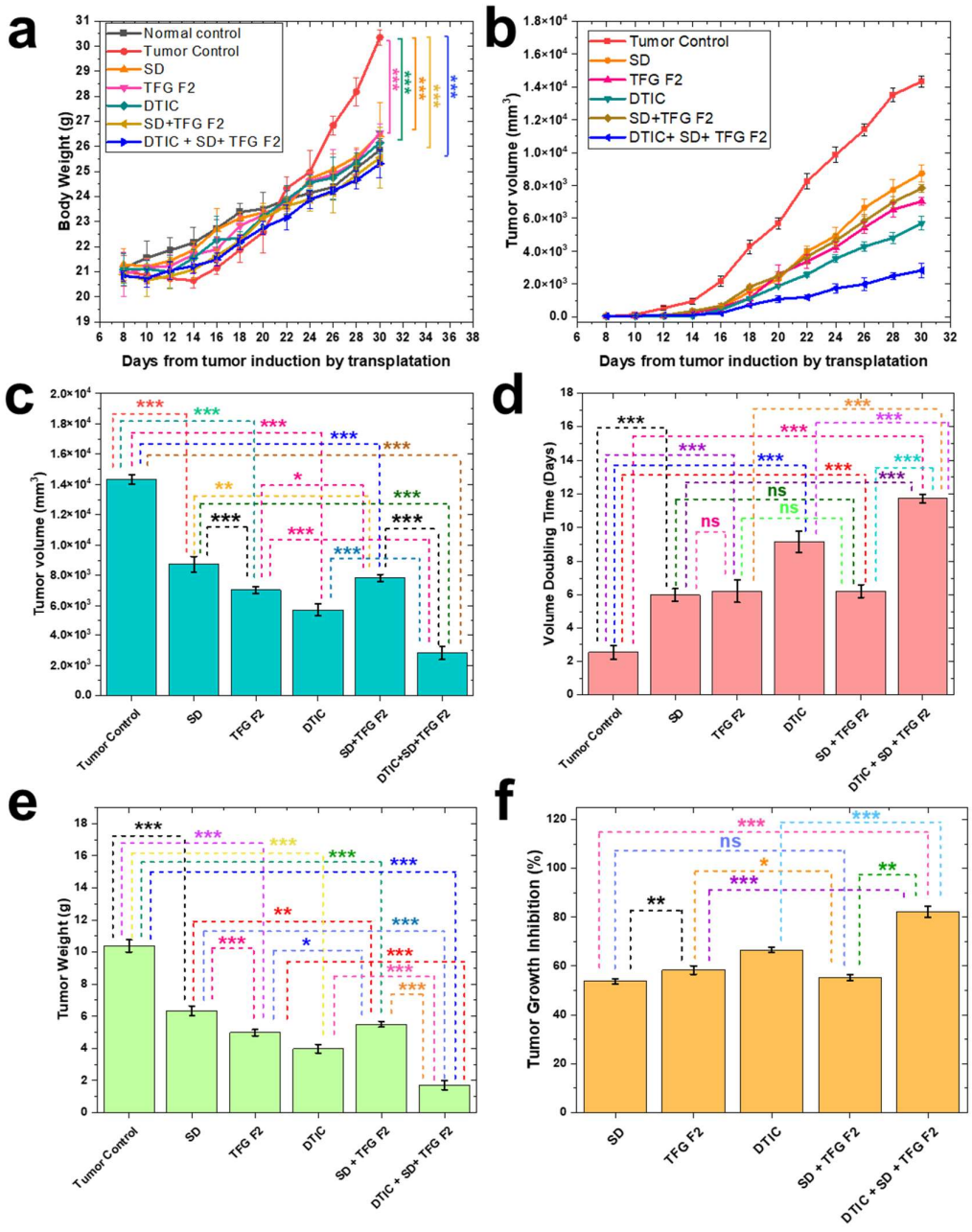

Figure 8. 2 Tumor regression analysis (a) changes in body weight, (b) tumor volume at an interval of two days after developed palpable tumor, (c) tumor volume on 30th day, (d) tumor volume doubling time (VDT) of various group, (e) tumor weight at 30th day, and (f) percent tumor growth inhibition (% TGI) of various groups at 30th day. The asterisks marks (*) represent the level of significance at $p<0.05$ in all cases. The statistical analysis was performed using one way ANOVA at followed by Tukey's test $p<0.05$ using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, California).

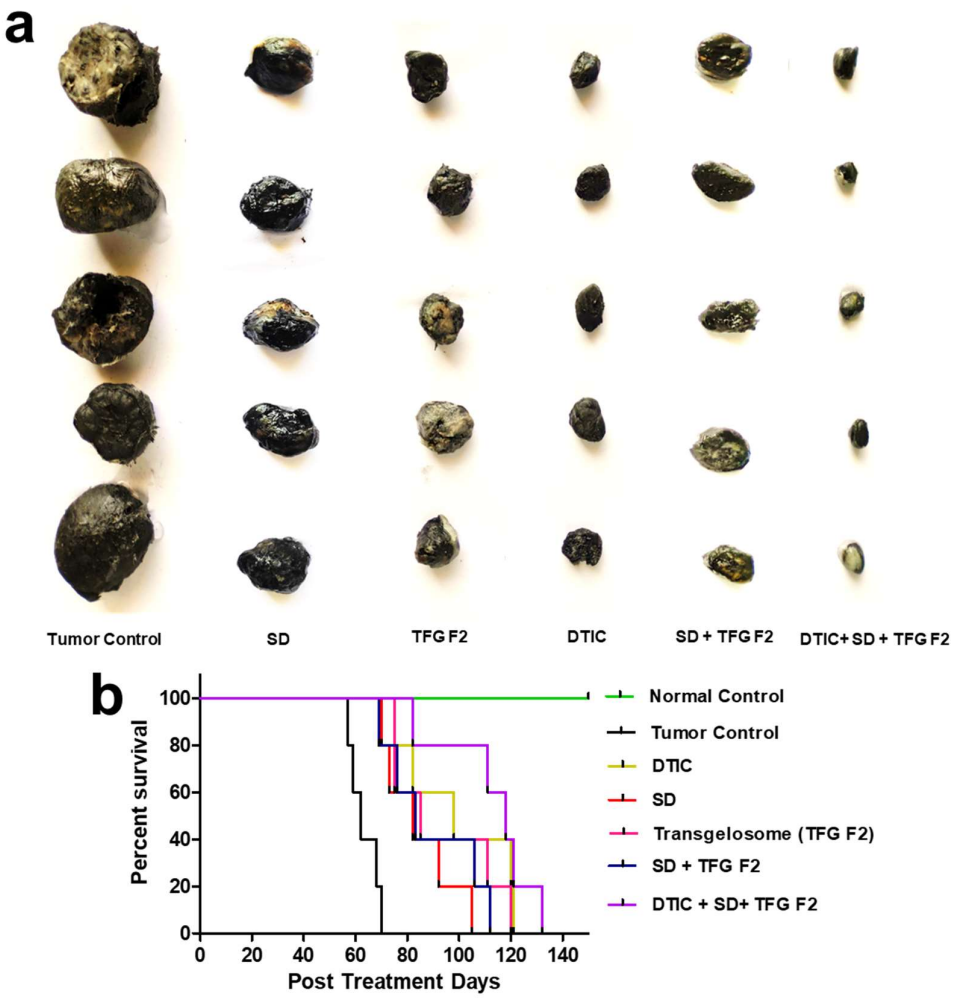

Figure 8. 3 Photograph of representative tumors from each group on 30th day

8.5 Conclusions

A combination of "SD + TFG F2" was found to be significantly more effective than SD alone at the same dose. The "SD + TFG F2," in combination with DTIC as an adjuvant therapy, was found to be therapeutically highly effective for the treatment of melanoma. Both the optimized formulations can be administered simultaneously to provide local and systemic effects for the effective treatment of melanoma.

8.6 Summary points

- Both the SD and TFG F2 were found to be effective for melanoma therapy either alone or as adjuvant therapy with DTIC.
- The TFG F2 showed stronger antitumor activity compared to optimized SD at the equivalent dose.
- Also, the TFG F2 demonstrated a good adjuvant with DTIC than that of SD for the effective treatment of melanoma.
- A combination of "SD + TFG F2" showed significantly more effective than SD alone at the same dose.
- The "SD + TFG F2," in combination with DTIC as an adjuvant therapy, was found to be therapeutically highly effective for the treatment of melanoma.