RESEARCH COMMUNICATION

# Carcinogenicity Testing of the Cosmetic Dye: D&C Red No. 36

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## Abstract

**D&C Red No. 36, a drug and cosmetic dye commonly used for coloring lipsticks, was evaluated for its carcinogenic potential in male and female Wistar rats. This dye has been shown to exhibit mutagenic activity towards *Salmonella typhimurium* TA 98 in the presence of S9 mix. In the present study, 50 male and 50 female rats in each group were given diets containing D&C Red No. 36 at 2 different concentrations, 1,000 ppm and 2,000 ppm, for 78 weeks and sacrificed at week 98.**

**It was found that dye treatment had no significant effect on the survival of either male or female animals as well as the body weight gain in males. However, body weight gain of treated females was slightly lower than that of the control group. Histopathological assessment demonstrated a number of benign and malignant tumors to have developed in various organs of both dye treated and control groups. In male rats, benign liver tumors were found at incidences of 16.7% and 18.8% of the low (1,000 ppm) and high (2,000 ppm) dose groups, respectively, similar to the 20% for the control group. Malignant tumors of the thyroid gland were observed only in the low dose and control groups, at 4.2% and 2%, respectively. In the high dose group, the incidences of lung, liver, urinary bladder and soft tissue cancers were 4.2%, 2.1%, 2.1% and 2.1%, respectively, only one soft tissue cancer being observed in a control group animal. In females, benign tumors were observed in the liver and mammary glands. The incidences of liver tumors in the low and high dose groups were 12.8% and 16%, respectively, and 6% in the control group. Values for mammary gland tumors were 10.6%, 10%, and 18% respectively. Malignant tumors were also observed in various other organs, including the uterus, lung, kidney, thyroid, thymus and salivary gland, but the incidences were very low (about 2-4%) and in dye treated male and female rats were not statistically different from those in the control animals.**

**The results of the present study thus demonstrated that D&C Red No. 36 at the concentrations of 1,000 ppm and 2,000 ppm in the diet is not carcinogenic either to male or female Wistar rats. While the occurrence of benign liver tumors in female rats may be related to dye treatment, the lack of any apparent dose-dependence or any statistically significant difference from the control group (P = 0.06) suggests that this is unlikely.**

**Key Words:** D&C Red No. 36 - carcinogenicity - cosmetic dyes - lipstick

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## Introduction

Results from various epidemiological studies revealed that 80-90% of all human cancers may be caused by environmental factors, and chemicals, either naturally or synthetically, are the predominant environmental carcinogens (Higginson, 1972; Higginson and Muir, 1973). These chemical carcinogens which are related to lifestyles include those in the diets, tobacco smoke, air, water, some drugs and cosmetics.

Lipstick usage, which is another widespread lifestyle, has received relatively little attention. Lipsticks contain various

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kinds of chemicals including azo dyes. Most of these azo dyes are drug and cosmetics quality, which are supposed to be used externally. Since the major portion of lipsticks are ingested (McCann, 1979), thus the safety of their ingredients should be obviously concern. Some of the azo dyes used as lipstick coloring, for example, D&C Orange Nos. 5 & 17 and D&C Red Nos. 7 & 36 have been shown to be mutagenic towards *Salmonella typhimurium* TA 98 (Brown et al., 1979; Muzzall and Cook, 1979; Green and Pastewka, 1980; Rojanapo et al., 1985). Moreover, D&C Red Nos. 9 and 19 have been reported to be carcinogenic in rats and mice (Goodman DG et al., 1984; Weinberger MA et al., 1985; Letter from US. FDA, 1985).

In our laboratory, we have tested the mutagenicities of lipsticks commonly used in Thailand, either produced locally or imported from western countries, towards *Salmonella typhimurium* TA 98 with and without metabolic activation and demonstrated that 44% (20 out of 46 samples) of lipsticks with orange or orange-red colors exhibited strong mutagenic activity, with 5 and 15 samples being mutagenic in the presence and absence of S9 mix, respectively. Furthermore, lipsticks mutagenic in the presence of S9 mix were found, by TLC analysis, to contain D&C Red No. 36 while those mutagenic in the absence of S9 mix contained D&C Orange No. 17, which exhibited strong mutagenicity in the presence and absence of S9 mix, respectively (Rojanapo et al., 1985). It is therefore of interest to evaluate the carcinogenic potential of these 2 strong mutagenic dyes. However, the US Food and Drug Administration announced that D&C Orange No. 17 was carcinogenic to animals and thereafter was banned since July 15, 1988, together with D&C Red Nos. 8, 9 and 19 (Goodman et al., 1984; Weinberger MA et al., 1985; Letter from US. FDA, 1985). We therefore tested the carcinogenic potential of only D&C Red No. 36 in male and female Wistar rats.

D&C Red No. 36 is monoazo dye. Azo compounds are the most common synthetic colorings used in foods, pharmaceuticals and cosmetics. The structure of this dye composed of the aniline ring and naphthalene ring (Fig. 1). Nitro groups on aromatic rings are known to be highly reactive and to be responsible for the carcinogenicity (Poirier and Weisburger, 1974; Miller, 1978; International Agency for Research on Cancer, 1978) and mutagenicity (Ames et al., 1975; Chiu, 1978) of a variety of such compounds. In this communication, we report results of our studies on the carcinogenicity of D&C Red No. 36 in male and female Wistar rats.

## Materials and Methods

*Chemical*

D&C Red No. 36 (C.I 12085, CAS No. 2814 -77- 9) or 1-azonaphth-2-ol-2-chloro-4-nitrobenzene was purchased from Warner Jenkinson Company, NJ, USA. The purity of the dye was not less than 95%.

*Animals and Diets*

Male and female Wistar rats weighing 40-60 g, age 4-5 weeks were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Animals were housed in suspended stainless steel mesh cages (5 animals per cage) in an air-conditioned room at $25 \pm 2\ ^0$C under a natural lighting. They were acclimatized in our laboratory for 1 week by giving normal powder diet (Institute of Food Research and Product Development, Kasetsart University, Bangkok, Thailand) and water ad libitum. The formula of the powder diet is shown in Table 1.

*Experimental Design*

A total of 300 rats (150 animals for each sex) were randomly divided by weight into 3 groups consisting of 50 males and 50 females in each group. Five animals were housed in a 58x28x18 cm stainless steel cage as mentioned above. Group I was assigned as the control group and was fed with normal powder diet ad libitum, while the other two groups (groups II and III) were assigned as experimental groups receiving powder diets containing D&C Red No. 36 at the concentrations of 1,000 ppm (low dose) and 2,000 ppm (high dose), respectively. Animals were given these diets throughout 78 weeks, weighed twice a month and they were necropsied when they died or became moribund. A total of 7 animals died during the first 40 weeks of the experiment and therefore excluded from this study. At 98$^{th}$ week, all surviving animals were sacrificed and various

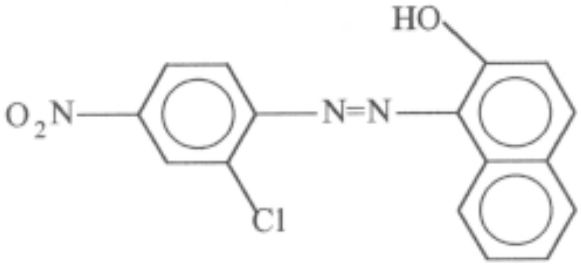


**Figure 1. Structure of D&C Red No.36**

**Table 1. Standard Powder Diet Formula**

| Composition | Amount (%) |
|---|---|
| Fish meal | 20 |
| Soy bean meal | 12 |
| Maize meal | 24 |
| Rice meal | 20 |
| Wheat bran | 15 |
| Saccharose | 2 |
| Soy oil | 2 |
| Mineral mixture | 3 |
| Vitamin mixture | 2 |

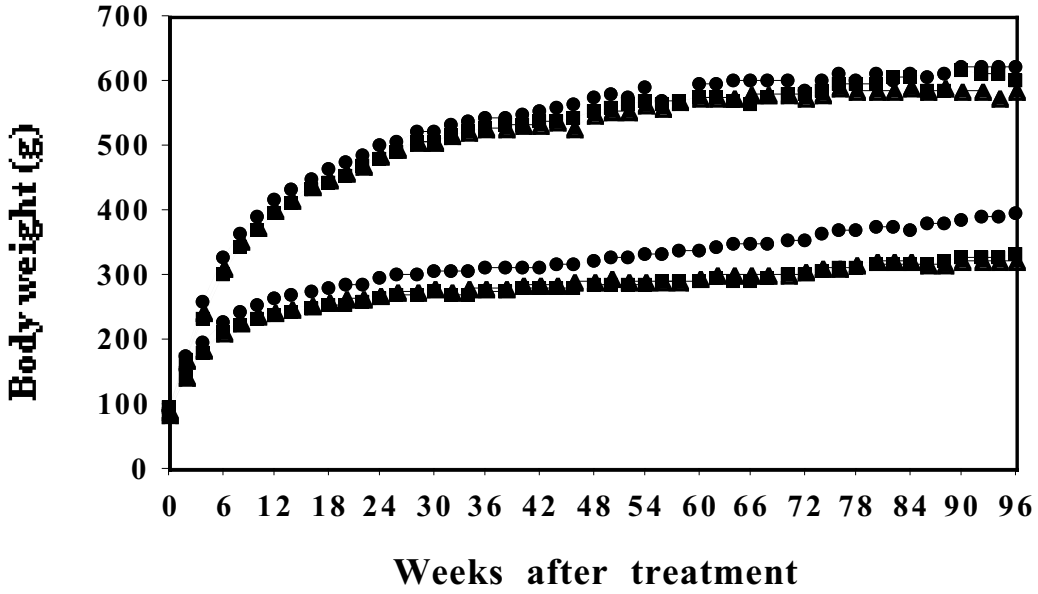


**Figure 2. Growth of Male and Female Rats Given Normal Powder Diet and Diet Containing D&C Red No. 36. Animals Receiving only Powder Diet were Served as Control (●——●) and the other 2 Groups were given Diets Containing 1,000 ppm (▲——▲) and 2,000 ppm (■——■) D&C Red No. 36.**

organs (i.e. lung, liver, spleen, kidney, urinary bladder, mammary gland, thyroid gland etc.) were fixed in buffered neutral formalin and routine histopathological examination of these tissues were performed after embedding in paraffin, sectioning and staining with H&E. The classification of both preneoplastic and neoplastic lesions was verified according to the criteria described previously (International Agency for Research on Cancer, 1990; 1994).

**Table 2. Percentage Survival of Rats Treated with D&C Red No. 36**

| Group | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | Initial No. | No. of survivors | % Survival | Initial No. | No. of survivors | % Survival |
| I (Control) | 50 | 50 | 100 | 50 | 50 | 100 |
| II (1,000 ppm) | 50 | 48 | 96 | 50 | 47 | 94 |
| III (2,000 ppm) | 50 | 48 | 96 | 50 | 50 | 100 |

**Table 3. Incidences of Benign Tumors in Rats Treated with D&C Red No. 36**

| Sex | Group | No. of effective rats | No. of rats bearing tumor (%) in Liver[a] | Thyroid gland[b] | Adrenal gland[c] | Bladder[d] | Mammary gland[e] |
|---|---|---|---|---|---|---|---|
| Male | I (Control) | 50 | 10 (20.0) | 1 (2.0) | 1 (2.0) | - | - |
| | II (1,000 ppm) | 48 | 8 (16.7) | 1 (2.1) | - | - | - |
| | III(2,000 ppm) | 48 | 9 (18.8) | - | 1 (2.1) | - | - |
| Female | I (Control) | 50 | 3 (6.0) | 1 (2.0) | - | 1 (2.0) | 9 (18.0) |
| | II (1,000 ppm) | 47 | 6 (12.8) | 1 (2.1) | - | - | 5 (10.6) |
| | III(2,000 ppm) | 50 | 8 (16.0) | 2 (4.0) | 1 (2.0) | - | 5 (10.0) |

[a]Include hyperplastic nodule and cystic cholangioma
[b]Include C-cell adenoma and follicular cell adenoma
[c]Cortical adenoma
[d]Transitional cell papilloma
[e]Include adenoma, fibroadenoma and fibroma

*Statistical Analysis*

The significant difference between treated and control groups of the body weight was analyzed by Student's *t*-test, whereas that of the tumor incidence was assessed by the statistical method described by Peto et al., 1980.

## Results

There was no difference in the growth rate and survival of male rats between control and both treated groups (Fig. 2 & Table 2). In female rats, dye treatment also did not affect the survival. However, the growth rate of both treated groups was only slightly, but significantly, lower than that of the control group (Fig. 2 & Table 2).

The incidence of benign tumors in various organs of rats fed control diet and that containing D&C Red No. 36 is shown in Table 3. Liver tumor was frequently found in both male and female rats. The tumor included hyperplastic nodule and cystic cholangioma. In male rats, the incidence of liver tumor was similarly occurred in the control and dye treated groups, being 20%, 16.7% and 18.8% in the control, low dose and high dose groups, respectively. In female rats, the incidence of liver tumor was dose-dependent relationship. The tumor was observed in only 6% of the control group, while observed in 12.8% and 16.0% of the low and high dose groups, respectively. However, the incidence in both treated groups was not significantly different from that in the control group (P=0.06).

Other types of benign tumor observed in male rats were thyroid and adrenal gland tumors, however, they were found in only about 2% in control and one treated groups. In female rats, mammary gland tumors which included fibroadenoma, adenoma and fibroma was observed in 18%, 10.6% and 10.0% of control, low and high dose groups, respectively. However, the difference between both treated and control groups was not statistically significant. Thyroid gland, adrenal gland and urinary bladder tumors were also found in the females of both control and treated groups, but the incidence was very low, only about 2-4%.

Table 4, summarizes the incidences and histopathological findings of malignant tumors in each group of animals. In male rats, thyroid tumor (follicular cell adenocarcinoma of papillary type) and soft tissue tumor (mixed malignant histiocytoma) were developed in 2% of the control group, while in the low dose group, only thyroid tumor which included C-cell carcinoma and follicular cell adenocarcinoma (papillary type) was observed in 4.2%. In the high dose group, various tumors, including lung, soft tissue, liver and urinary bladder tumors were observed, but only 4.2%, 2.1%, 2.1% and 2.1%, respectively. However, the incidence of all types of malignant tumors observed in dye treated male rats was not significantly different from that in the control group.

In the females, malignant tumors were also observed in various organs, including lung, thyroid gland, thymus gland, salivary gland, uterus and kidney of both control and treated groups. However, the incidence was very low, only 2-4% and the difference between treated and control groups was not statistically significant.

## Discussion

In the present study, the carcinogenicity of D&C Red No. 36 was evaluated by mixing the dye in the diet in order to imitate the route of human exposure, i.e. ingestion along with the lipsticks while taking meals. McCann has stated that woman usually ingests approximately 0.03 mg lipstick/kg body weight/day (McCann, 1979). We have estimated the amount of D&C Red No. 36 in the lipsticks, based on the TLC results and the mutagenicities of lipsticks and the dye, and found that lipsticks may contain this dye ranging from 0.25-4% and some of them may contain the dye as high as 6% (Rojanapo et al., 1985). Thus, a woman would ingest the dye approximately 0.075-1.8 mg/kg/day.

Administration of the dye at the concentration of 1,000

**Table 4. Incidences of Malignant Tumors in Rats Treated with D&C Red No. 36**

| Sex | Group | No. of effective rats | No. of rats bearing tumor (%) in | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Lung[a] | Thyroid gland[b] | Thymus gland[c] | Soft tissue[d] | Salivary gland[e] | Uterus[f] | Others |
| Male | I (Control) | 50 | - | 1 (2.0) | - | 1 (2.0) | - | - | - |
| | II (1,000 ppm) | 48 | - | 2 (4.2) | - | - | - | - | - |
| | III(2,000 ppm) | 48 | 2 (4.2) | - | - | 1 (2.1) | - | - | 2 (4.2)[1] |
| Female | I (Control) | 50 | - | 1 (2.0) | 2 (4.0) | - | 1 (2.0) | 1 (2.0) | 1 (2.0)[2] |
| | II (1,000 ppm) | 47 | - | - | - | - | - | 1 (2.1) | - |
| | III(2,000 ppm) | 50 | 1(2.0) | 1 (2.0) | 1 (2.0) | - | 1 (2.0) | - | 1 (2.0)[3] |

[a]Include adenocarcinoma and mesenchymal tumor
[b]Include follicular cell adenocarcinoma and C-cell carcinoma
[c]Malignant thymoma
[d]Include fibrosarcoma and mixed malignant fibrous histiocytoma
[e]Mixed carcinoma
[f]Histiocytic sarcoma
[1]Include hepatocellular carcinoma, transitional cell carcinoma of urinary bladder
[2]Adenocarcinoma of mammary gland
[3]Wilm's tumor

and 2,000 ppm in the diet had no effect on the survival of the animals, although it caused a decrease in the body weight gain of female rats, but not the males. This may be partly due to reduced food consumption as noted in the female groups only. The diet containing dye may have unpalatable taste.

Results in this study demonstrated that D&C Red No. 36 induced benign liver tumors in female rats with a dose dependent relationship, although the difference between control and treated groups was not statistically significant (P=0.06). This result seems to be in agreement with those reported previously for D&C Red Nos. 9, 19 and D&C Orange No. 17. D&C Red No. 9 and No. 19 induced liver tumor in the males and females, respectively, while D&C Orange No. 17 induced this tumor in female rats (Goodman et al., 1984; Weinberger et al., 1985; Letter from US. FDA, 1988; International Agency for Research on Cancer, 1993). The development of liver tumor in male rats in this study was quite high (about 17-20%), but the incidence in the treated groups were similar to that in the controls.

The mechanism of liver tumor induction by D&C Red No. 36 which is an indirect mutagen, i.e. requiring metabolic activation before exerting its mutagenicity, may be through the reduction of the azo group of the dye. Reduction of aromatic azo and nitro compounds by metabolic activation in the body has been shown to result in the formation of compounds with toxic, carcinogenic and mutagenic activities (Walker, 1970).

It is also interested to note that the incidence of benign mammary gland tumors were lowered in the treated females when compared to the controls. The explanation of this phenomenon is not known. However, azo dye has been reported to induce drug detoxification enzymes (De Long et al., 1986), thus it is conceivable that azo dye treatment may lead to an increase in the excretion of some toxic compounds including those contaminated in the environment and thereby resulting in a decrease in the incidence of spontaneous tumors.

Malignant tumors have been also observed in various organs, including lung, thyroid gland and thymus gland of both dye-treated male and female animals. However, the incidence of these tumors was very low and not significantly different from that in the control group. These malignant tumors that occurred in both male and female rats might be spontaneous tumors in aging animals (Ratcliffe, 1949; Roe, 1965), since this study took about 2/3 of life span (2 years) of rats.

Thus, from all results in the present study it can be concluded that D&C Red No. 36 may not be carcinogenic to both male and female Wistar rats when giving orally in the diet at the concentration of 1,000 and 2,000 ppm. However, this dye may involve in the development of benign liver tumor in female rats since the occurrence of tumor appeared to be treatment related. Similar experiment to be performed in mice and different strain of rats is recommended in order to confirm results in the present study.

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