The Journal of Applied Pharmacology, **13**, 113-117(2005)

# *In Vivo* Anti-tumor Activity of 3-Methyl-6-allylthiopyridazine in Nude Mice Xenografted with Hep-G2 Hepatocarcinoma

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(Received June 7, 2005; Accepted June 22, 2005)

**Abstract** – Organosulfur compounds have been shown to exert an anti-cancer activity. In an attempt to develop novel chemopreventive and anti-cancer agents for liver cancer, we synthesized allylthiopyridazine derivatives. We have previously shown that allylthiopyridazine derivatives exert inhibitory effects on proliferation, invasion and migration of SK-Hep-1 hepatocarcinoma cells *in vitro*. The *in vivo* anti-tumor effect of 3-methyl-6-allylthiopyridazine, named as K6, was also reported. In this study, we further investigated the preclinical anti-cancer efficacy of K6 for hepatocarcinoma using nude mice xenografted with Hep-G2 hepatocellular carcinoma cells. K6 (20-100 mg/kg, orally administered everyday for 30 days) markedly decreased the tumor volume of Hep-G2 cell-transplanted nude mice as evidenced by ultrasonographic and plethysmographic analyses. The inhibitory effect on tumor volume was lower than that exerted by doxorubicin (2 mg/kg, intravenously injected) which was used as a positive control. This study shows that K6 efficiently suppresses xenograft tumor growth, revealing K6 as a potential anti-cancer agent for suppressing *in vivo* progression of liver cancer. Given that hepatocarcinoma is among the most prevalent and lethal malignancies and there is no effective treatment to date, our study may contribute to the potential drug development for liver cancer.

**Keywords** □ Allylthiopyridazine, hepatocarcinoma, xenograft

## INTRODUCTION

Organosulfur compounds which are components of garlic oil have been shown to exert various biological activities such as antibacterial, antifungal, antithrombotic, cholesterol-lowering, antineoplastic and hepatoprotective activities (Kwon, 2003). Diallylsulphides inhibit tumor cell proliferation and suppress chemically-induced carcinogenesis in experimental animals. A synthetic sulfur-containing compounds including oltipraz and sulindac exert chemopreventive and hepatoprotective effects (Clapper, 1998; Rao *et al.*, 1995; Goluboff *et al.*, 1999).

In spite of its early discovery, pyridazine has not gained much attention compared with the structurally similar compounds, pyrimidine and pyrazine. Recently, studies have been performed to reveal the biological and pharmacological activities of pyridazine and its derivatives (Cho and Kim, 1998; Bandoh *et al.*, 1995). In an attempt to develop novel chemopreventive and anti-cancer agents, we synthesized a series of allylthiopyridazine derivatives designated as K compounds by introducing allylthio moieties into heterocyclic ring of pyridazine (Kwon, 2000; Kwon and Hyun, 2000). We have previously demonstrated that 3-alkoxy-6-allylthiopyridazines, the compounds having alkoxy residues in their *para* position, show antiproliferative effect on SK-Hep-1 hepatocarcinoma cells by inducing apoptosis (Jung *et al.*, 2001). The K compounds inhibited invasion and migration in SK-Hep-1 cells possibly *via* MMP-2 downregulation (Lee *et al.*, 2003). Our previous study also revealed that these allylthiopyridazine derivatives prevented hepatoxicity induced by aflatoxin $B_1$ in rats (Shin and Kwon, 2003). The *in vivo* anti-tumor effect of an allylthiopyridazine compound was reported by using human xenograft implanted into nude mice (Chai *et al.*, 2004). In this study, we further confirmed the preclinical anti-cancer efficacy of K6 (3-methyl-6-allylthiopyridazine) for hepatocarcinoma. Doxorubicin was used as a positive control since doxorubicin, when administered intravenously, was shown to be efficient in inhibiting the tumor growth in a new orthopotically impaired human hepatocellular carcinoma model (Labonte *et al.*, 2000). Here, we show that K6 efficiently suppresses the tumor growth of nude mice transplanted with Hep-G2 hepatocarcinoma-derived

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tumor, suggesting that this compound can be used as a potential anti-cancer agent for suppressing *in vivo* progression of liver tumor.

## Materials and methods

### Allylthiopyridazine derivative, K6

An allylthiopyridazine derivative was synthesized using pyridazine as the parent nucleus. 3-Methoxy-6-chloropyridazine was obtained from 3,6-dichloropyridazine. 3-Methoxy-6-allylthiopyridazine was then synthesized from halide and allylmercaptane (Kwon and Hyun, 2000; Jung *et al.*, 2001) and was designated as K6 (Fig. 1).

### Experimental animals

Seven-week old male Balb/C nude mice weighing 16-18g were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed in filter-topped cages (26W × 42L × 18H). Animal room was maintained for constant temperature (22 ± 2°C), light (12 hr light/dark cycle) and humidity (50 ± 10%). All laboratory food pellets and beddings were pre-sterilized by autoclaving.

### Xenograft transplantation

After three passages, confluent Hep-G2 cells were washed twice with Hank's balanced salt solution (HBSS, Gibco BRL, Grand Island, NY, USA), trypsinized with 0.25% trypsin in HBSS, and washed twice with fresh culture medium. Hep-G2 cells ($1 \times 10^8$ cells/mouse) in 0.1 ml HBSS were injected subcutaneously in the flank of nude mice using a 26-gauge needle. Solid tumor fragments (3 × 3 × 3 mm) were transplanted in the flank of nude mice using a troca. Size of the transplanted tumor derived from Hep-G2 cells was measured using a digital caliper (Tokyo, Japan) twice a week.

### Administration of K6 and doxorubicin

The nude mice transplanted with tumor fragment were randomly distributed into experimental groups (five mice per each group) when the tumor volume reached 100 $mm^3$. K6 was orally administered everyday for 30 days. Doxorubicin (2 mg/kg) was intravenously injected at days 5, 9, 13, 19, 24. For control group, nude mice were administered corn oil everyday for 30 days.

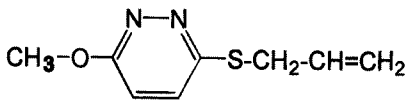


**Fig. 1.** The chemical structure of 3-methyl-6-allylthiopyridazine (K6).

### Measurement of tumor volume by ultrasonography

The mean tumor volume was measured by an ultrasonography. The largest and the deepest diameters of tumor were measured at the end of treatment using an ultrasonography (Sonoace 8800, Seoul, Korea).

### Measurement of tumor weight and volume by plethysmography

Solid tumor was removed and weighed at the end of the experiment. Tumor volume (V) in $mm^3$ was measured by a plethysmometer (LETICA, Spain) and calculated according to the following equation: $V = AB^2/2$ where A and B are the largest and the smallest or the deepest tumor diameters in mm, respectively.

### Statistical analysis

The results are presented as the means ± S.D. and the significance of difference between the mean of each group was analyzed using one-way analysis of variance (ANOVA) followed by a Dunnet's t-test correction, paired t-test, and linear regression analysis.

## RESULTS

In order to investigate the *in vivo* anti-cancer efficacy of K6 on the tumor originated from hepatocarcinoma, we used human tumor xenografts implanted into nude mice. Hep-G2 hepatocarcinoma cells were injected subcutaneously in the flank of nude mice. Solid tumors were observed at 14-16 days after injection. Success rate of developing solid tumors was 75.6%. The solid tumor was evenly fragmented (3 × 3 × 3 mm) and the tumor fragments were transplanted in the flank of nude mice. The nude mice were used for experiments when the tumor volume reached 100 $mm^3$. Using these animals, we investigated the inhibitory effect of K6 on the tumor growth by performing an ultrasonographic analysis to measure the final tumor volume. As shown in Fig. 2, the tumor size was markedly reduced by treatment of K6 (100 mg/kg) compared to the control group. The tumor-reducing effect of K6 was lower than that exerted by doxorubicin (2 mg/kg). The mean tumor volume of control group measured at the final day of experiment was 2,156 $mm^3$ (Table 1). Administration of K6 resulted in a significant reduc-

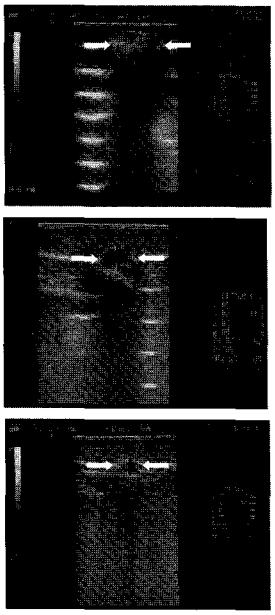


**Fig. 2.** K6 decreases tumor volume measured by ultrasonography. Nude mice bearing solid tumor derived from Hep-G2 cells were treated with K6 (100 mg/kg) or doxorubicin (2 mg/kg) as a positive control. The length and depth of tumor (marked by arrows) in the animals were measured at the final day of experiment.

tion of tumor volume compared with control group in a dose-dependent manner: the mean tumor volumes of animals treated with 20, 50 and 100 mg/kg of K6 were 722 $mm^3$, 695 $mm^3$ and 544 $mm^3$, respectively. Doxorubicin administration (2 mg/kg) decreased the mean tumor volume to 393 $mm^3$.

The final tumor volume was also measured by plethysmography (Table I). A significant decrease in tumor volume was observed in the animals treated with 100 mg/kg of K6 which was comparable to that exerted by doxorubicin (2 mg/kg). The

**Table I.** Effects of K6 on the final tumor volume in nude mice bearing solid tumor derived from Hep-G2 cells.

| | Tumor Volume ($mm^3$) | |
|---|---|---|
| | Ultrasonography | Plethysmography |
| Control | 2156.00 ± 913.98 | 740 ± 0.08 |
| Doxorubicin (2 mg/kg) | 392.88 ± 199.83** | 460 ± 0.18* |
| K6 (20 mg/kg) | 722.30 ± 415.11* | 600 ± 0.20 |
| K6 (50 mg/kg) | 695.00 ± 143.69* | 570 ± 0.15 |
| K6 (100 mg/kg) | 544.30 ± 158.24* | 450 ± 0.17** |

Doxorubicin was used as a positive control. The values are represented as the mean ± S.D. (n = 5). *,**Significantly different from control at $p < 0.05$ and $p < 0.01$, respectively.

data were plotted as a bar graph (Fig. 3A). These results, along with the data on the inhibition of the final tumor weight of animals upon treatment of K6 (Fig. 3B), clearly demonstrate that K6 efficiently suppresses the tumor of nude mice xenografted with solid tumor derived from Hep-G2 hepatocarcinoma.

## DISCUSSION

Hepatocellular carcinoma is one of the most lethal malignancies and there is no effective preventive measure to date. We have previously shown that allylthiopyridazine derivatives exert inhibitory effects on proliferation, invasion and migration of SK-Hep-1 hepatocarcinoma cells *in vitro* (Jung *et al.*, 2001; Lee *et al.*, 2003). Using human xenograft implanted into nude mice, we previously demonstrated the *in vivo* anti-tumor effect of an allylthiopyridazine compound, K6 (Chai *et al.*, 2004). In this study, we further investigated the preclinical anti-cancer efficacy of K6 (3-methyl-6-allylthiopyridazine) for hepatocarcinoma and showed that the administration of K6 significantly slowed xenograft tumor growth.

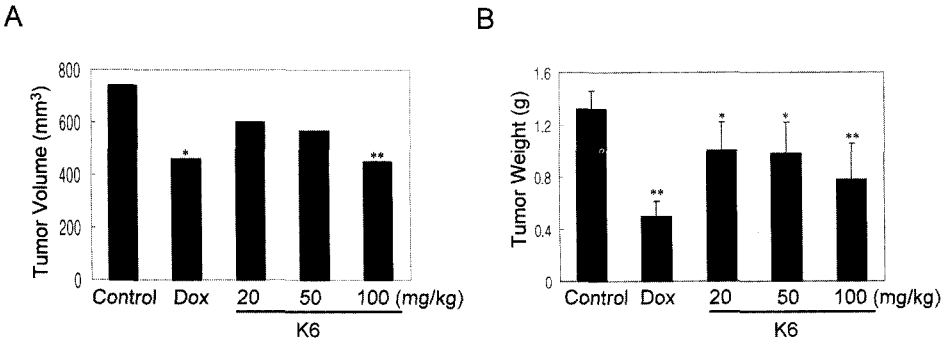


**Fig. 3.** K6 inhibits tumor growth *in vivo*. Nude mice bearing solid tumor derived from Hep-G2 cells were treated with various concentrations of K6 or 2 mg/kg of doxorubicin (Dox) as a positive control. ***A***, Tumor volume was measured by plethysmography at the final day of experiment. ***B***, Tumor weight was measured at the final day of experiment (Chai et al., 2004). The values are represented as the mean ± S.D. (n=5). *,**Significantly different from control at $p < 0.05$ and $p < 0.01$, respectively.

Human tumor xenografts implanted subcutaneously into immunodeficient mice provide valuable means to evaluate potential anti-tumor drugs in preclinical study and are applicable for studying many different types of human malignancies (Langdon and Hendricks, 1994). Although activity within a particular histological type of the xenograft generally does not predict for clinical activity in the same tumor (Kelland, 2004), hepatocarcinoma cell lines have been proven to be useful for xenograft to investigate the anti-cancer efficacy for liver cancer (Goluboff *et al.*, 1999; Yang *et al.*, 1997). Recently, efforts have been made to develop more clinically relevant models by the use of orthotopic transplantation of tumor material in rodents (Bibby, 2004).

Doxorubicin is one of the anthracycline antibiotics with an anti-tumoral activity. Doxorubicin has been used widely in treating various tumors. The multiple potential mechanistic actions of doxorubicin include the inhibition of activities of DNA topoisomerase II and III, inhibition of helicases, alteration of membrane structure and function and generation of free radical (Elka *et al.*, 2002). Treatment of doxorubicin can cause side effects such as cardiotoxicity, neurotoxicity and reproductive system toxicity. Anti-neoplastic drugs used in chemotherapy, such as adriamycin, methotrexate, doxorubicin and cyclophosphamide, are reactive compounds that can cause failures of germ cell production (Imahie *et al.*, 1995; Williams et al., 2000). It has been demonstrated that whereas doxorubicin-treated mice show testis atrophy and deletion of germ cells in gross and histopathological findings, the administration of K6 did not cause *in vivo* toxicity of the testis, suggesting that K6, unlike doxorubicin, had no side effect on reproductive system of experimental animals (Chai *et al.*, 2004). Furthermore, oral administration with K6 at high doses up to 100 mg/kg for 30 days did not affect the normal gain of body weight while treatment with doxorubicin resulted in a significant decrease in body weight (Chai *et al.*, 2004).

The present study showed that K6 exerted an efficient *in vivo* anti-tumor activity in the animals xenografted with hepatocarcinoma-derived tumor. Although the efficacy of K6 was lower than that of doxorubicin, the less adverse effect of K6 suggests that this compound might be a useful anticancer agent for therapy of human liver cancer.

## ACKNOWLEDGMENTS

This work was supported by a 2004 Research Grant from Duksung Women's University, Seoul, Korea. We are grateful to Biotoxtech Co. Ltd (Korea) and Ms. Jane Hwang for technical assistance.

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