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Effect of DW2282 on the Induction of Methemoglobinemia, Hypoglycemia or WBC Count and Hematological Changes

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DW2282,(S)-(+)-4-phenyl-1-[1-(4-aminobenzoyl)-indoline-5-sulfonyl]-4,5-dihydro-2-imidazolone hydrochloride, is a new anticancer agent which is thought to exhibit a characteristic mechanism of action in the inhibition of tumor growth. In this study, we estimated the toxicities of DW2282 in mice. When mice were orally dosed for five consecutive days at the dosages of 50, 100 and 150 mg/kg, DW2282 did not induce methemoglobinemia and hypoglycemia at any of these doses. However, increased ALT and AST values were observed in the 150 mg/kg dosing group, and white blood cells (WBC) were significantly decreased at all doses. However, the changes in WBC count, ALT and AST immediately reversed after the cessation of drug administration. In addition, we found that DW2282 did not cause an increase in hemolysis in human blood. Taken together, these data suggested that DW2282 may have a relatively low level of toxicity, and that there may be a quick recovery from any toxicity it does produce.

Key words: DW2282, Methemoglobin, Blood glucose, WBC, ALT, AST

INTRODUCTION

DW2282 is a new possible antitumor agent, which has been modified from diarylsulfonylureas, sulofenur (Mohamadi *et al.*, 1992; Howbert *et al.*, 1990). Sulofenur (Fig. 1) has been reported to have broad antitumor activities in several solid tumor models (Houghton *et al.*, 1990a; Houghton *et al.* 1990b; Houghton *et al.*, 1989), but its distinct mechanism of action has not yet been clearly deduced (Houghton *et al.*, 1990b; Howbert, 1991; Ehlhardt *et al.*, 1997; Houghton and Houghton, 1996; Sosinski *et al.*, 1994; Boder *et al.*, 1989; Houghton *et al.*, 1990c). However, the development of sulofenur as an antitumor agent was discontinued during clinical trials because of the severe methemoglobinemia resulting from its toxic metabolite, p-chloraniline. (Taylor *et al.*, 1992; Talbot *et al.*, 1993; Hainworth *et al.*, 1989). To overcome the limitation of sulofenur, we have synthesized 4-phenyl-1-(1-benzoylindoline-5-sulfonyl)-imidazolidinone analogs (Jung *et al.*, 1996a; Jung et al., 1996b; Jung et al., 1998). DW2282 (Fig. 1) exhibits cytotoxic activity and an antitumor effect (Jung *et al.*, 1998), and has been determined to be a candidate for the development of a novel anticancer agent.

In the study described here, we tested whether DW2282 affects methemoglobin in the same way that sulofenur did (Taylor *et al.*, 1992; Talbot *et al.*, 1993; Hainworth *et al.*, 1989), and whether it lowers blood glucose levels, which is characteristics of other sulfonylurea derivatives (Pfeifer *et al.*, 1981). We also tested whether DW2282 has the same myelosuppressive effect which has been shown in most other anticancer drugs (Blum and Carter, 1974; Carter, 1975). In addition, we searched for other toxicities of DW2282, in our investigation as to the developmental possibility of DW2282 as

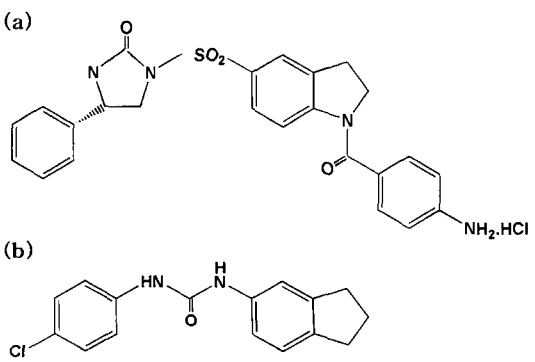

Fig. 1. The structures of DW2282 (a) and sulofenur (b).

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an oral anticancer drug.

MATERIALS AND METHODS

Animals

Five male CD-1 mice (Charles River Laboratories, Japan) in each group were used to collect blood at designated times. Animals were acclimated for at least one week to the animal facilities, which were maintained at 23 ± 1°C, 55 ± 5% humidity, 10-18 circulation/hour and 12 hrs cycle of light/dark. Feed and water were freely accessible to the mice.

Chemicals

DW2282 was provided by the synthetic division of the Central Research Laboratories of Dong-Wha Pharm. Ind. Co. Ltd. in Korea. The DW2282 was dissolved in propylene glycol (PG) as a vehicle, and orally administered to mice at doses of 50, 100, 150 mg/kg for five consecutive days. The vehicle was orally administered at 5 ml/kg. For the assay of methemoglobin, 150 mg/kg of aniline, used as a positive control, was administered to mice, i.p., on the day before their sacrifice. Unless indicated, all chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO).

The measurement of methemoglobin

This test was based on the absorbance reduction of methemoglobin by the formation of cyan-methemoglobin at 630 nm (Tietz et al., 1976; Evelyn and Malloy, 1938). 100 μl of blood was hemolysed using 3.9 ml of distilled water, and diluted with an equal volume of 0.15 M phosphate buffered saline (PBS). 3 ml of diluent was added to tube-1 and tube-2, and then 100 μl of 20% $K_3Fe(CN)_6$ was mixed with the hemolysate of tube-2 only. Following a two minute reaction, the absorbances of the reactants of tube-1 and tube-2 were measured at 630 nm and designated as tube-$1_{initial}$ and tube-$2_{initial}$. After the reaction with 100 μl of 5 % KCN, the absorbance was measured at 630 nm and designated as tube-1_{final} and tube-2_{final}. The percentage of methemoglobin in each blood sample was calculated as follows.

$$\%\text{Methemoglobin} = [(\text{Tube-}1_{initial} - \text{Tube-}1_{final})/(\text{Tube-}2_{initial} - \text{Tube-}2_{final})] \times 100$$

Determination of blood glucose level

This experiment was performed using the method proposed by Keston *et al.* (1956) and modified by Beach and Turner (Beach and Turner, 1958). This method was based on the principle that glucose was oxidized by glucose oxidase to hydrogen peroxide, which oxidized O-dianisidine to a brown product in the presence of peroxidase. In brief, 5 μl of plasma and standard glucose solution (100 mg/dL) was mixed with 95 μl of distilled water and 1 ml of coloring agent containing 40 μg/ml O-dianisidine. 5.0 units/ml glucose and 1.0 units/ml horeseradish peroxidase were added and reacted at 37°C for 30 minutes. Absorbance was measured at 450 nm with an ELISA reader (Bio-Tek, Winooski, VT). The glucose concentration was calculated as follows.

$$\text{Glucose in plasma (mg/dl)} = (A_{plasma} / A_{standard\ solution}) \times 100$$

Blood collection

Blood samples were collected by orbital puncture on designated days. Plasma was immediately separated by centrifugation of the blood and stored at -70°C for the determination of blood glucose, alanine aminotransferase (ALT) and aspartate aminotrasferase (AST) level. For the measurement of methemoglobin and WBC, blood was used within three hours of collection.

White blood cell (WBC) count

DW2282 was orally administered to the mice for five consecutive days, and the number of WBCs was counted during the experimental period as follows (Miescher and Gerade, 1966): The blood was diluted and suspended in a WBC-diluting solution containing 2 ml of glacial acetic acid, 1 ml of crystal violet (1% aqueous) and 97 ml of distilled water, which was filtered before use. The WBC-diluting solution could lysis the erythrocytes and stain the WBCs. The WBCs were counted under a light microscope, WBC counts are described as cells/ μl by multiplying by the dilution factor.

Analysis of ALT and AST

The levels of ALT and AST were measured using the method proposed by Reitman and Frankel (1957), who used the principles that the sub-strates, DL-alanine and L-asparagic acid are changed by the catalysis of ALT and AST, respectively, to pyruvic acid. The pyruvic acid then reacts with 2,4-dinitrohydrazine for color formation to be quantitated by absorbance. In brief, 50 μl plasma was reacted with the substrate of ALT for 30 minutes or AST for 60 minutes. 2,4-dini-trohydrazine was then added and reacted for 20 minutes. 0.4 N NaOH was added to interrupt the reaction and the absorbance was measured at 495 nm in 60 minutes. The amounts of ALT and AST were calculated from the standard curve using linear-regression method.

Hemolytic activity of DW2282 in vitro

Various doses of DW2282 (0.4-10 μg/ml) were incubated with freshly collected human blood from healthy

volunteers for designated times at 37°C. Aliquots of the supernatants obtained after centrifugation were diluted 1:100 with 0.9% NaCl and the concentration of hemoglobin was determined by calculating the difference between the absorbance at 577 nm and 561 nm. Total hemolysis (100%) was obtained by incubating the erythrocytes in water containing 0.02 % triton X-100 at a 1:1 (v/v) ratio (Schwender *et al.*, 1996).

Statistical analyses

All experiments were performed three times and data were represented as mean ± S.D.. The differences between the DW2282-treated groups and vehicle-treated controls were analyzed by the Students t-test.

RESULTS

Clinical signs, histopathological observations and body weights

DW2282 was administered to the mice for five consecutive days, and the body weight of each animal was checked daily from the first. When the mean body weight of the DW2282-exposed group was evaluated during the dosing period, no statistical differences were found compared to the control group (data not shown). No clinical signs were observed in the 50 and 100 mg/kg DW2282-treated groups. However, incoordination and piloerection were observed in the 150 mg/kg DW 2282-treated group. Organ weights, including the liver, lung, spleen, kidney and stomach were unchanged. In addition, no gross lesions of the liver, spleen and kidney were found either in dead or terminally sacrificed animals (data not shown).

Effect of DW2282 on methemoglobin level

We investigated whether DW2282 raised methemglobin levels. As shown in Fig. 2, DW2282 did not induce methemoglobin at any dose ranges when compared to the control group. The methemoglobin level in the total hemoglobin of the vehicle-treated control was 3.37 % and those in the 50, 100 and 150 mg/kg DW2282-treated groups were 2.71, 3.2 and 3.68 %, respectively. The differences between the control and DW2282-treated groups were not significant. However, the methemoglobin level in the aniline-treated positive control group increased significantly by one and a half times that of the control ($p<0.05$).

Effect of DW2282 on blood glucose level

We tested whether blood glucose levels were affected by DW2282. As seen in Fig. 3, DW2282 did not induce hypoglycemia at any of the dose ranges after

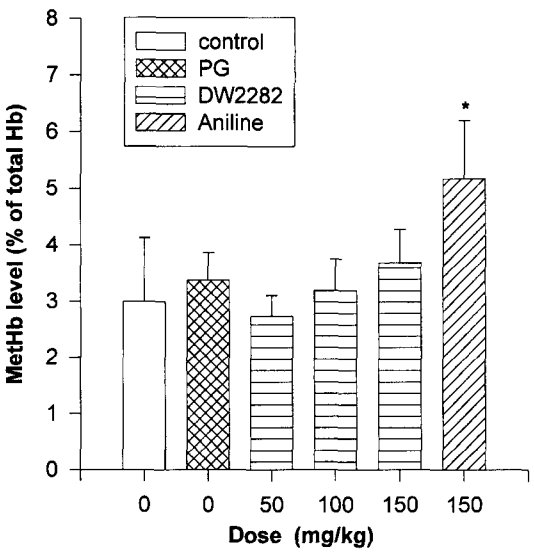

Fig. 2. Effect of DW2282 on methemoglobin level. Blood was obtained from mice after the oral administration of DW 2282 for five days. It was hemolysed, reacted with 5% KCN for the formation of cyanmethemoglobin, and the absorbance was measured at 630nm. $p<0.05$, significantly different from the PG-administered group.

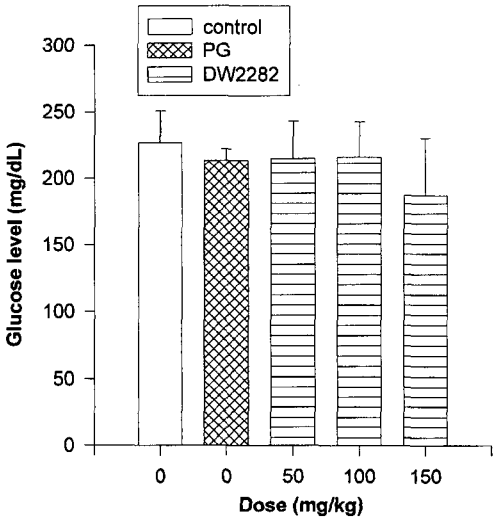

Fig. 3. Effect of DW2282 on blood glucose level. Plasma was obtained after the oral administration of DW2282 for five days. It was reacted with O-dianisidine for the formation of brown color, which was measured at 450 nm.

administration for five consecutive days. The glucose level in the control group was 227 mg/dl, and those in the 50, 100 and 150 mg/kg DW2282-treated groups were 215, 216 and 187 mg/dl, respectively. There were no significant differences between the control and DW 2282-treated groups ($p<0.05$).

Effect of DW2282 on WBC count

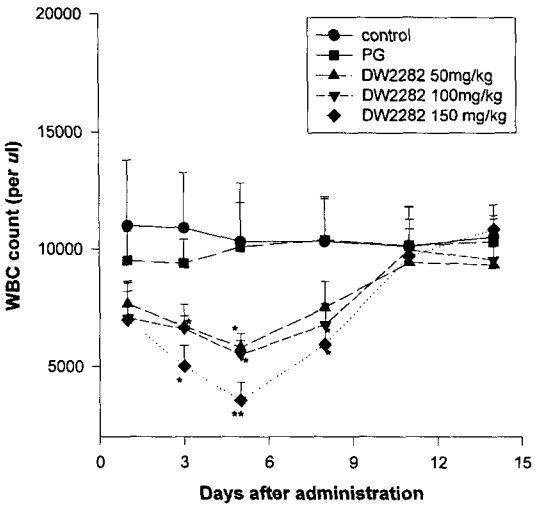

Fig. 4. Effect of DW2282 on WBC count. Blood was obtained by orbital puncture of mice after the oral administration of DW2282 for five days. Leukocytes were stained with 0.01% crystal violet and their number was enumerated under a light microscope. Three mice in the DW2282 150 mg/kg treated group were dead at day eight (2 of 5) and day ten (1 of 5), respectively. $p<0.05$; $p<0.01$, significantly different from PG-administered group.

We also measured whether DW2282 reduced the number of leukocytes, which is an indication of myelosuppression. The WBC count in all DW2282-treated groups gradually decreased from the first DW2282-administration day (day 0) to the last administration (day 4) ($p<0.05$; $p<0.01$), and continued to decrease after DW2282 discontinuation (Fig. 4). The number of leukocytes at day six rapidly increased, and returned to the same status as that of the control group after day 11. Two mice in the 150 mg/kg DW2282-treated group were dead at day 8, and one mouse from this group was dead at day 10.

Effect of DW2282 on ALT and AST level(s)

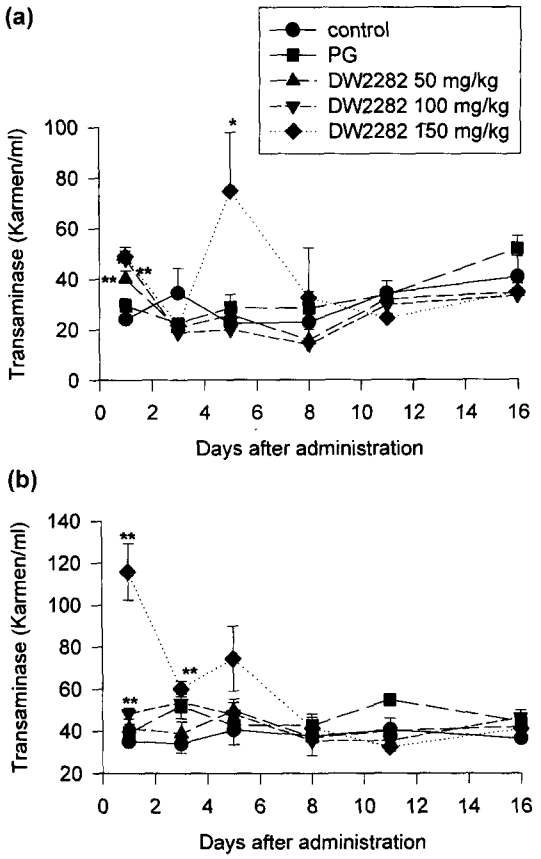

Fig. 5. Effect of DW2282 on ALT (a) and AST (b) levels. Plasma was obtained from mice after the oral administration of DW2282 for five days. It was then incubated with substrates of ALT and AST, and the color formation by 2,4-dinitrohydrazine was measured at 495 nm. Three mice in the DW2282 150 mg/kg treated group were dead at day eight. $p<0.05$; $p<0.01$, significantly different from PG-administered group.

ALT and AST levels were measured following five multiple doses of DW2282. As evident in Fig. 5, 100 and

Table I. Hemolytic activity of DW2282 on human blood

Sample	hemoglobin concentration (%)				
exposure time (hr)	1	3	5	24	48
control	0.122	0.207	0.782	0.641	0.229
	(0.041)	(0.225)	(0.313)	(0.317)	(0.114)
DW2282 10 μg/ml	0.162	0.272	0.704	0.697	0.229
	(0.000)	(0.195)	(0.078)	(0.128)	(0.114)
DW2282 2 μg/ml	0.203	0.195	0.469	0.641	0.152
	(0.041)	(0.039)	(0.078)	(0.128)	(0.066)
DW2282 0.4 μg/ml	0.162	0.233	0.469	0.669	0.343
	(0.000)	(0.079)	(0.078)	(0.290)	(0.114)

DW2282 was incubated with human blood for various lengths of time. Hemoglobin concentration was measured by the difference between absorbance at 577 nm and at 561 nm. Data are represented as mean ± S.D. of triplicate experiments. S.D. was expressed by the numbers in parentheses.

150 mg/kg of DW2282 significantly increased ALT and AST after the initiation of administration, but 50 mg/kg caused no changes when compared to the control group ($p<0.05$; $p<0.01$). It is not yet clear why the levels of ALT and AST activity fluctuated during the DW2282 treatment. In any case, the elevated levels of ALT and AST returned to normal after day 8. Two mice in the 150 mg/kg DW2282-treated group were dead at day 8.

Hemolytic activity of DW2282 on human blood

When DW2282 was incubated with human blood to predict human toxicities, there were no significant changes in the percent of hemolysis in the DW2282-treated group at up to 48 hrs, as compared to the control group, in which hemolysis was less than 1% (Table I). In contrast, the percentage of hemolysis in rat blood did increase after 48 hrs of exposure to DW2282 (data not shown).

DISCUSSION

DW2282 is one of the diarylsulfonylurea derivatives synthesized by the Dong-Wha Pharmaceutical Company in Korea. It has shown excellent *in vitro* cytotoxicity and *in vivo* antitumor activity against human tumor cells (Jung *et al.*, 1998). We performed preliminary testing of its fundamental toxicities such as methemoglobinemia and hypoglycemia to be specified by DW2282s structure itself. It has been previously reported that the major metabolite of sulofenur, 4-chloroaniline, may induce methemoglobinemia and hemolytic anemia which could account for the animal and human toxicities (Taylor *et al.*, 1992; Talbot *et al.*, 1993; Hainworth *et al.*, 1989). From the results of the measurement of methemoglobinemia and hypoglycemia, it has been thought that the structure modification of sulofenur to DW2282 is satisfactory, and that DW2282 may have different characteristics than other diarylsulfonylurea derivatives.

Until now, it has been known that most anticancer drugs, including adriamycin, decrease the number of leukocytes after administration (Carter, 1975). In this study, we found that DW2282 also reduced the number of leukocytes. However, the recovery of the number of leukocytes to normal was very fast after the last administration of DW2282. These results show that DW 2282 could be a good anticancer drug, which has a reversible effect on the differentiation of the immune cells which play an important role in the body defense against microorganisms and tumor cells.

DW2282 would be developed as an oral anticancer drug. When administered p.o., most drugs are metabolized by the hepatic enzymes in the liver into hydrophilic materials or into active metabolites which could damage liver tissue. As noted by Cornelius (1987), ALT and AST are the major indicators of liver damage. Therefore, we measured ALT and AST levels in order to determine the effect of DW2282 on the liver after administration for five consecutive days. ALT and AST levels increased at the initiation of DW2282 administration, but rapidly returned to normal after its discontinuation. This shows that the hepatotoxicity of DW2282 is reversible, and it also shows that DW2282 is a possible long-term anticancer drug. To preliminarily investigate whether DW 2282 affects the hemolysis of blood in vitro, DW2282 was incubated with human blood for appropriate periods of up to 48 h. DW2282 did not cause an increase in the hemolysis of human blood even after 48 hrs of exposure, as compared to the results in rat blood (data not shown). This shows that DW2282s toxicities in humans may be lower than those in rats.

We suggest that DW2282 could be an oral anticancer drug with a relatively low level of toxicity, and that there may be a quick recovery from any toxicity it does produce, when compared to other antitumor agents presently being used. However, the death of some of the animals in the 150 mg/kg DW2282-treated group shows that this drug may have a dose-limited therapeutic application.

REFERENCES CITED

Beach, E. F. and Turner, J. J., An enzymatic method for glucose determination in body fluids. *Clin. Chem.*, 4, 462-469 (1958).

Blum, R. H. and Carter, S. K., Adriamycin : A new anticancer drug with significant clinical activity. *Ann. Internal Med.*, 80, 249-259 (1974).

Boder, G. B., Beasley, F. W. and Grindey, G. B., Mechanism of action studies with LY186641. *Proc. Amer. Assoc. Cancer Res.*, 30, abstr. 2203 (1989).

Carter, S. K., Adriamycin : A review. *J. Natl. Cancer Ins.*, 55, 1265-1274 (1975).

Cornelius, C. E., A review of new approaches to assessing hepatic function in animals. *Vet. Res. Commun.*, 11, 423-441 (1987).

Ehlhardt, W. J., Woodland, J. M., Toth, J. E. , Ray, J. E. and Martin, D. L., Disposition and metabolism of the sulfonylurea oncolytic agent LY295501 in mouse, rat and monkey. *Drug Metabol. Dispos.*, 25, 701-708 (1997).

Evelyn, K. A. and Malloy, H. T., Micro-determination of oxyhemoglobin, methemoglobin and sulfhemoglobin in single sample of blood. *J. Biol. Chem.*, 126, 655-662 (1938).

Hainworth, J. D., Hande, K. R., Satterlee, W.G., Kuttesch, J., Johnson, D.H., Grindey, G. B., Jackson, L.E. and Greco, F. A., Phase I clinical study of N-[(4-chlorophenyl)-amino]carbonyl-2,3-dihydro-1H-indene-5-sulfonamide (LY186641). *Cancer Res.*, 49, 5217-5220 (1989).

Houghton, P. J., Bailey, F. C., Germain, G. S., Grindey, G. B., Witt, B. C., Houghton, J. A., N-(5-Indanylsulfonyl)-N-(4-chlorophenyl)urea, a novel agent equally cytotoxic to non-proliferating human colon adenocarcinoma cells. *Cancer Res.*, 50, 318-322 (1990a).

Houghton, P. J., Bailey, F. C., Houghton, J. A. , Murti, K. G., Howbert, J. J. and Grindey, G. B., Evidence for mitochondrial localization of N-(4-methylphenylsulfonyl)-N-(4-chlorophenyl)urea in human colon adenocarcinoma cells. *Cancer Res.*, 50, 664-668 (1990b).

Houghton, P. J., Bailey, F. C., Germain, G. S., Grindey, G. B., Howbert, J. J. and Houghton, J. A., Studies on the cellular pharmacology of N-(4-methylphenylsulfonyl)-N -(4-chlorophenyl) urea. *Biochem. Pharmacol.*, 39, 1187-1192 (1990c).

Houghton, P. J. and Houghton, J. A., Antitumor diarylsulfonylureas: novel agents with unfulfilled promise. *Invest. New Drugs*, 14, 271-280 (1996).

Houghton, P. J., Houghton, J. A., Meyers, L., Cheshire, P., Howbert, J. J. and Grindey, G. B., Evaluation of N-(5-indanylsulfonyl)-N-(4-chlorophenyl)-urea against xenografts of pediatric rhabdomyosarcoma. *Cancer Chemother. Pharmacol.*, 25, 84-88 (1989).

Howbert, J. J., Sulofenur. *Drug Future.*, 16, 517-520 (1991).

Howbert, J. J., Grossman, C. S., Crowell, T. A., Rieder, B. J., Harper, R.W., Kramer, K.E., Tao, E.V., Aikins, J., Poore, G. A., Rinzel, S. M., Grindey, G. B., Shaw, W.N. and Todd, G. C., Novel agents effective against solid tumors: the diarylsulfonylureas. Synthesis, activities and analysis of quantitative structure-activity relationships. *J. Med. Chem.*, 33, 2393-2407 (1990).

Jung, S. H. Song, J. S., Lee, H. S., Choi, S. H. and Lee, C. O., Synthesis and Evaluation of cytotoxicity of novel arylsulfonylimidazolidinones containing sulfonylurea pharmacophore. *Arch. Pharm. Res.*, 19, 570-580 (1996a).

Jung, S. H. Song, J. S., Lee, H.S., Choi, S. H. and Lee, C. O. Synthesis and Evaluation of cytotoxicity of novel arylsulfonylimidazolidinones. Bioorg. Med. Chem. Lett. 6, 2553-2558 (1996b).

Jung, S. H., Lee, H. S., Song, J. S., Kim, H. M., Han, S. B., Lee, C. W., Lee, M., Choi, D. R., Lee, J. A., Chung, Y. H., Yoon, S. J., Moon, E. Y., Hwang, H. S., Seong, S. K. and Lee, D.K., Synthesis and antitumor activity of 4-phenyl-1-arylsulfonyl imidazolidinones. *Bioorg. Med. Chem. Lett.*, 8, 1547-1550 (1998).

Keston, A. S., Specific colorimetric ezymatic analytical reagents for glucose, Abstract of papers, 129th Meeting, ACS, Dallas, Taxas, April, p31C (1956).

Miescher, P. A. and Gerarde, H. W., A one-step method for counting leukocytes and platelets. *Am. J. Clin. Pathol.*, 46, 576-581 (1966).

Mohamadi, F., Spees, M. M. and Grindey, G. B., Sulfonylureas: A new Class of Cancer Therapeutic Agents. *J. Med. Chem.*, 35, 3012-3016 (1992).

Pfeifer, M. A., Halter, J. B., Beard, J. C. and Porte, D. J., Differential effects of tolbutamide on first and second phase insulin secretion in non-insulin-dependent diabetes mellitus. *J.Clin.Endoclinol. Metab.*, 53, 1256-1262 (1981).

Reitman, S. and Frankel, S., A colorimetric method for the determination of serum glutamic axaloacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.*, 28, 56-64 (1957).

Schwender, R. A., Horber, D. H., Odermatt, B. and Schott, H., Oral antitumor activity in murine L1210 leukemia and pharmacological properties of liposome formulations of N^4-alkyl derivatives of 1-*B*-D-arabinofuranosylcytosine. *J. Cancer Res. Clin. Oncol.*, 122, 102-108 (1996).

Sosinski, J., Thakar, J. H., Germain, G. S., Dias, P., Harwood, F. C., Kuttesch, J. F. and Houghton, P. J., Cross-resistance of antitumor diarylsulfonylureas, and collateral sensitivity to mitochondrial toxins in a human cell line selected for resistance to the antitumor agent N-(5-indanylsulfonyl)-N-(4-chlorophenyl)urea. *Mol. Pharmacol.*, 45, 962-970 (1994).

Talbot, D. C., Smith, I. E., Nicolson, M. C., Powles, T. J., Burton, D. and Walling, J., Phase II trial of the novel sulfonylurea sulofenur in advanced breast cancer. *Cancer Chemother. Pharmacol.*, 31, 419-422 (1993).

Taylor, C. W., Albert, D. S., Peng, Y. M., McCloskey, T. M., Matzner, M., Roe, D. J., Plezia, P. M., Grindey, G. B., Hamilton, M. and Seitz, D., Antitumor activity and clinical pharmacology of sulofenur in ovarian cancer. *J. Natl Cancer Inst.*, 84, 1798-1802 (1992).

Tietz, N. W., Fundamentals of clinical chemistry pp 414. W. B. Saunders Co., Philadelphia (1976).