Exp Oncol 2009
31, 1, 22–26

Experimental ONCOLOGY

SYNTHESIS AND EVALUATION OF DIMETHYL TIN 4-CYCLOHEXYL THIOSEMICARBAZONE AS A NOVEL ANTITUMOR AGENT

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Aim: **To develop a rationally designed new organotin compound namely dimethyl tin 4-cyclohexyl thiosemicarbazone (D4-t) and evaluate its putative antitumor activity.** ***Methods:*** **Starting from 4-cyclohexyl thiosemicarbazone, a three step synthetic procedure was followed to obtain the title compound.** ***In vivo*** **lymphocyte activation property of the compound at three different doses was assayed by measuring the blastogenesis. Concanavalin A (ConA) was used as standard mitogen for murine T cells stimulation** ***in vivo*****. Also, the synthesis of DNA by the activated lymphocytes was measured after injecting the D4-t. The lymphocyte activation property and antitumor efficacy of D4-t were assessed in Sarcoma-180 (S-180) bearing mice. The organization of lymphoid cells was studied in the histological preparations of spleen and mesenteric lymph node. Tumor neutralization assay (Winn assay) was conducted to examine whether immune responses were associated with the manifestation of antitumor efficacies of this compound in S-180** ***in vivo*****. The DNA synthesis inhibitory effect of the compound in S-180 cells was studied** ***in vitro*****, and was found significant ($P < 0.001$).** ***Results:*** **Different doses of the new compound caused differential response of blastogenesis and DNA synthesis. In comparison to ConA, the title compound showed a good number of blast cells at its optimum dose of 5 mg/kg. It caused maximum synthesis of DNA by the lymphoid cells. In histological preparations, the gradual transformation of lymphocytes into blasts was observed without any visible toxicity. Winn assay revealed that 5 mg/kg of D4-t was able to reduce tumor mass without severe toxicity. This organotin compound also inhibits the synthesis of DNA in S-180 tumor cells in comparison to Platin10 and ConA.** ***Conclusion:*** **The title compound has the lymphocyte activation property and stimulates immune response of the lymphoid cells, which in turn express the antitumor activity without any significant toxicity. Results indicate promising therapeutic potential of D4-t.**

Key Words: **new organotin compound, synthesis, lymphocyte activation, antitumor activity.**

The earliest reports on the therapeutic use of the metals or metal-containing compounds in cancer and leukemia were dated back to 19th century. Until 1960s, the knowledge about mechanism of their antitumor activity was limited due to the technological restrictions and lack of scientific approach for their exploration. The discovery of the inorganic complex cis-diammine-dichloroplatinum (II) (cisplatin) led to the development of other types of non-organic cytostatic drugs for the treatment of tumors. Numerous other metal compounds that contained platinum, other platinum metals, and even non-platinum main group metals, including tin, were then shown to be effective against tumors. In the field of organometallic chemistry organotin compounds shares a very important prospective. The organotin compounds or stannanes are chemical compounds based on the tin with hydrocarbon substituents. In recent years, organotin compounds have drawn much attention as antitumor agents [1–3]. The $R_2Sn(IV)^{2+}$ compounds exhibit maximal antitumor activity with low toxicity, are the type $R_2SnX_2L_2$ (X = halogen, pseudo halogen and L= O- or N-donor ligands) [4–7]. Diorganotin(IV) compounds, R_2SnCl_2 are often tetrahedral, and structurally similar with cisplatin. A large number of such complexes have been tested for antitumor activity [3, 8]. Investigations of thiosemicarbazone Sn(IV) complexes have been carried out by many researchers [2, 9, 10]. It has been observed that several di- and triorganotin(IV) species were active against various types of cancer cells [5, 11]. The compounds usually realize their effect by interacting with the nucleic acid base [12]. A number of positive results have been reported in this direction [3, 7, 13]. Organotin compound has antiproliferative ability and antitumor activity on mammalian cells both *in vitro* and *in vivo* [3, 7, 8, 13]. Also, in Japan works regarding the immune response induced by implantation of tumor cells was further stimulated by D-fraction of Maitake mushroom have been carried out [14].

In the present study, we studied the potential antitumor activity of a novel rationally designed compound dimethyl tin 4-cyclohexyl thiosemicarbazone (D4-t), which have ONS (oxygen/ nitrogen/sulphur) donors in its structure to develop an appropriate "bite angles". The compound is expected to coordinate with DNA base pairs. The synthesis, lymphocyte activation and evaluation of D4-t anticancer activity were performed and discussed.

MATERIALS AND METHODS

Chemical synthesis. The ligand 4-cylohexyl thiosemicarbazone was prepared from cyclohexyl thiosemicarbazide and salicyl aldehyde as described [15]. Then methanol solution of dimethyltin dichloride and sodium hydroxide solution (50% excess) were mixed to prepare dimethyltin oxide [10, 16]. After that, condensation of dimethyltin oxide (1 M) and the ligand

Received: July 25, 2008.
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Abbreviations used: CHN – carbon hydrogen nitrogen; D4-t – dimethyl tin 4-cyclohexyl thiosemicarbazone; LN – lymph nodes; MLN – mesenteric lymph node; ONS – oxygen nitrogen sulphur; S-180 – sarcoma180; SP – spleen.

(1 M) in benzene (100 ml) was performed by Dean Stark method for 8 h [10, 16]. The prepared D4-t was purified from pet ether and benzene.

IR spectroscopy (FTIR-8300-Shimadzu, KBR optics using nujol mull) and ^{1}H NMR spectra (^{1}H NMR: Bruker 300-DPX, δ values, TMS as internal standard) of D4-t were measured. Release of radioactivity was counted in liquid scintillation counter (Model no. LKB 1209 Rack-Beta).

In vivo screening. All *in vivo* experiments were conducted according to the rules and regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Sarcoma-180 (S-180) cells were obtained from the National Center for Cell Sciences (Pune, India) and maintained in Swiss albino male mice (6–7 weeks old, 24 ± 2 g) by intra peritoneal (i. p.) transplantation of 1.0×10^{6} cells/0.2 ml physiological saline/animal [17]. Mice were divided in groups as control (untreated), D4-t treated and ConA / Platin 10 treated. At least 6 animals were used for a particular group in an experiment. Physiological saline containing 0.2 ml of Freund's incomplete adjuvant (Sigma-Aldrich Chemicals Pvt. Ltd., India) was used as vehicle for drug administration by i.p. injection. Compounds in respective doses were administered to each mouse in treated groups as per the experiment performed. Group 1: The control groups received equal volumes of vehicle. Group 2: Mice treated with D4-t in respective doses through i.p. route. Group 3: Concanavalin A (ConA) (Sigma-Aldrich Chemicals Pvt. Ltd., India) dissolved in sterilized distilled water before intravenous (i. v.) injection was used as positive control. For evaluation of antitumor activity cisplatin injection (Platin10) (Cadila Pharmaceuticals Ltd., India) was used as positive control.

In vivo lymphocyte activation. The degree of activation by ConA of lymphocytes of secondary lymphoid organs and peripheral blood is considered as the measure of *in vivo* lymphocyte activation [18, 19]. Mice were injected with three different doses of D4-t on day 0: 1 mg/kg, 5 mg/kg and 10 mg/kg of body wt of mice. Cell suspensions from different lymphoid organs such as spleen (SP), mesenteric lymph node (MLN) and other lymph nodes (LN) were prepared. The peripheral blood (PB) was on Ficoll and Hypaque solution (Sigma-Aldrich Chemicals Pvt. Ltd., India) for separation of lymphocytes [18, 19]. Then D4-t-stimulated blast transformation was measured. The dose of 50 µg of ConA in 0.1 ml/animal was chosen for lymphocyte activation. The rate of blast transformation of lymphocytes of lymphoid organs was recorded at 24 h up to 96 h [18].

The process of activation and blast transformation of lymphocytes were evaluated by the rate of DNA synthesis, which was measured by the rate of ^{3}H-thymidine incorporation into DNA. The optimal dose of ConA for DNA synthesis was 10 µg in 0.1 ml/animal [18]. So this dose was used in this case apart from using three different doses of D4-t injected on day 0. The assay was made at 24 h up to 96 h after injection. Cell suspensions of SP were adjusted to 1.0×10^{6} / 0.1 ml after the addition of ^{3}H-thymidine (activity 10 µCi each) dissolved in 0.01 ml sterile saline. Using the standard method of incubation for 8 h followed by precipitation with 10% chilled trichloroacetic acid, the release of radioactivity was recorded with the help of liquid scintillation counter [18, 20].

Evaluation of antitumor activity. S-180 tumor cells (1.0×10^{6} cells/animal) were s. c. implanted in normal Swiss albino mice on day 0. At least 6 animals were used for a particular group, divided as control (untreated) and treated (D4-t, ConA / Platin 10) in an experiment.

S-180 bearing animals treated with D4-t on day 1 by i. p. route for this experiment (optimal dose of 5 mg/kg) were killed, their SP and MLN were isolated at different time points after day 1 (24, 48, 72 and 96 h), and fixed in Bouin's fixative. Histological sections were made and stained with Delafield's haematoxylin and eosin. The histopathological changes were evaluated by microscopy. In control, the experiments were performed without any drug injection and ConA was used as standard [21].

In Winn or tumor neutralization assay, D4-t (5 mg/kg dose by i. p.) and ConA (50 µg in 0.1 ml/animal by i. v.) were injected on day 1 after injecting S-180 on day 0. Then on day 5, SP cell suspension from ConA and D4-t treated mice or S-180 bearing control mice was mixed with S-180 tumor cells (1 : 1) and inoculated into normal mice. ConA treated, D4-t treated and control mice were weighed daily as described in [14].

To study the inhibitory effects of D4-t on DNA synthesis in S-180 *in vitro* ^{3}H-thymidine incorporation assay was used as described in [22]. Tumor cells were removed at the 7th day after tumor transplantation, washed twice in Hank's balanced salt solutions, resuspended in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, streptomycin (100 µg/ml) and penicillin (100 units/ml), followed by viable cell count. To the cells (1.0×10^{6}/0.1 ml) with ^{3}H-thymidine (10 µCi each) D4-t was added at concentration of 0.0016 µM and cells were incubated at 37 °C for 30 min and 60 min. As controls, the same concentrations of ConA and Platin10 were used.

Statistical analysis. Values were expressed as the mean ± SD. Experimental results were analyzed by Student's *t*-test. $P < 0.05$ was considered as the level of significance for values obtained for treated groups, compared with the control group.

RESULTS

The pathway of D4-t synthesis is presented on Fig. 1. Melting point (M. pt.) of D4-t was 97–98 °C. The yield of the dark yellow coloured solid compound was 80%. The calculated Carbon Hydrogen Nitrogen (CHN) percentage (%) was 45.3, 5.4, 9.9 and the observed CHN % was 44.8, 5.2, 9.1 respectively. IR (cm^{-1}) values was 1600 (CH=N), 968 (C-S), 530 (Sn-O), 478 (Sn-C). ^{1}H NMR (δ ppm, $CDCl_3$) of D4-t was 0.85 (s, 6H, $2\times CH_3$), 1.35 (m, 11H, C_6H_{11}),

4.7 (d,1H,NH), 6.77 (m,2H, aromH), 7.1 (d,1H,aromH), 7.26 (m,1H,aromH) and 8.6 (s,1H, CHN).

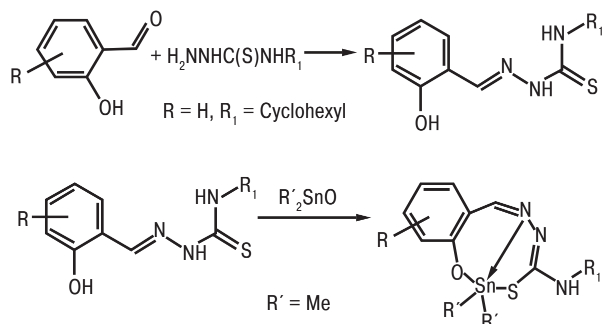

Fig. 1. The pathway of D4-t synthesis

In vivo activation of lymphocytes with different doses of D4-t at different time intervals was examined. The dose wise effect of ConA is presented on Fig. 2, *a*. The dose wise effects of D4-t are presented on Fig. 2, *b–d*. The peak of blastogenesis of cells from all sources (SP, LN, MLN and PB) irrespective of D4-t dose was effectively reached at 72 h. The responses were almost at the same level in 24 h for all three studied D4-t doses. The blast cells became more vacuolated or exhausted at 72 h up to 96 h. The increase in the percentage of vacuolated cells corresponded to the 10 mg/kg of D4-t (see Fig. 2, *d*). The significance of the difference between the groups ($P < 0.01$) was found.

Patterns of DNA synthesis by SP cells upon treatment by D4-t and ConA are presented on Fig. 3. With all three studied D4-t doses, maximum incorporation of ^{3}H-thymidine was observed at 48 h, and the index decreased between 48 h up to 96 h time points ($P < 0.001$). The rate of DNA synthesis was higher for 5 mg/kg dose and almost reached the ConA value.

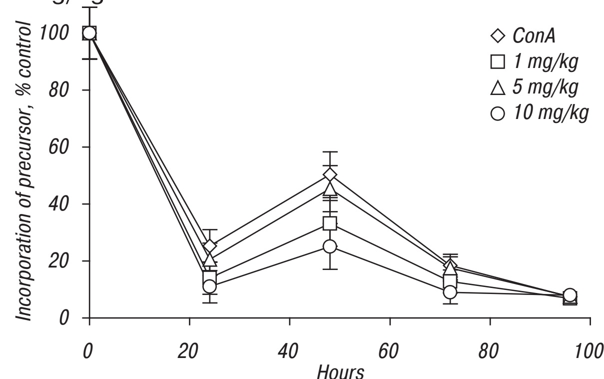

Fig. 3. Pattern of ^{3}H-Thymidine incorporation by splenic lymphocytes at different time points after *in vivo* stimulation by ConA (10 µg) and D4-t (1 mg/kg, 5 mg/ kg, and 10 mg/ kg)

The *in vivo* antitumor activity of D4-t in S-180 was determined following the treatment of animals at the dose of 5 mg/kg.

In histological preparations of SP and MLN the gradual transformation of the lymphocytes into blasts was observed. Within 24 h of the treatment, SP cells of D4-t treated animals were loosely packed in comparison to control mice (S-180 bearing untreated mice). A higher percentage of blasts in MLN than in SP were recorded within 24 h. By 48 h most of the MLN cells differentiated into blast cells (data not presented).

In Winn assay the weight of tumor bearing mice, treated by D4-t increased from the day 1 to day 14, but after day 15 decreased compared with the weight of control animals and reached the value shown for ConA treated animals (Fig. 4). Significant regression ($P < 0.01$) was observed in mice inoculated with the

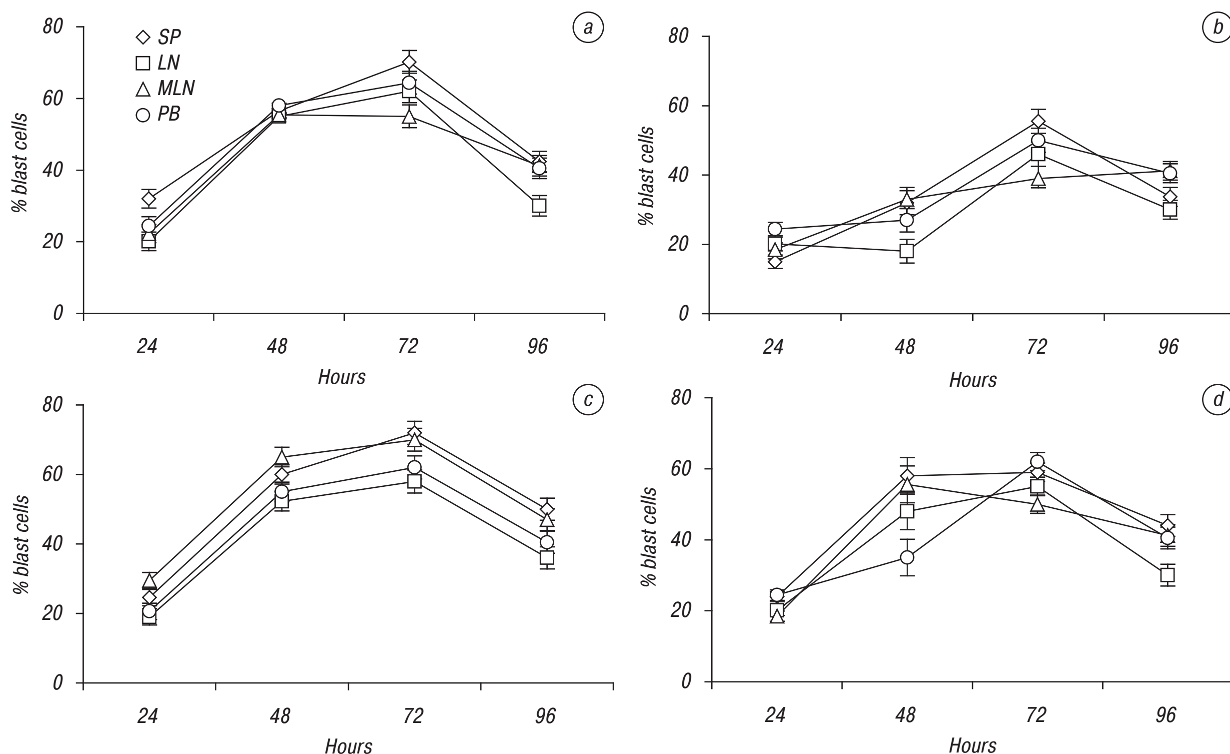

Fig. 2. Kinetics of blastogenesis of lymphocytes treated by ConA at a dose of 50 µg in 0.1 ml/animal (*a*), by D4-t at a doses of 1 mg/kg body weight (*b*), of 5 mg/kg (*c*) and 10 mg/kg (*d*)

mixture of tumor cells and SP cells obtained from D4-t pre-treated mice.

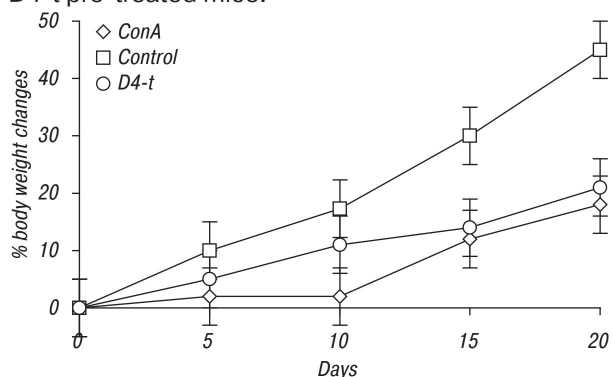

Fig. 4. Body weight changes of mice treated by D4-t, ConA and control mice in tumor neutralization assay (Winn assay)

To ascertain whether drug-induced tumor growth inhibition was achieved due to the inhibitory effect of D4-t on DNA synthesis, we performed DNA synthesis assay. The untreated S-180 cells demonstrated an almost linear pattern of ^{3}H-thymidine uptake over a period of 60 min (Fig. 5). Simultaneous exposure of tumor cells to 0.0016 μM D4-t resulted in gradual and marked inhibition of ^{3}H-thymidine uptake ($P < 0.001$). The effect was almost equal to that in Platin10-treated cells, but greater than for ConA-treated cells. Thus, at the end of 60 min incubation Platin10, ConA and D4-t exhibited 95%, 66% and 80% of DNA synthesis inhibition compared with control untreated cells, respectively (see Fig. 5).

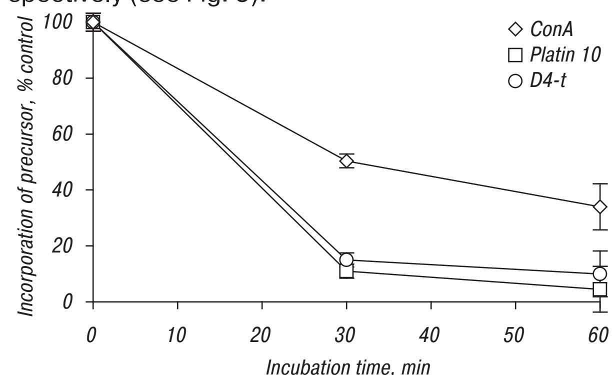

Fig. 5. Effect of Platin10, D4-t and ConA on DNA synthesis by S-180 tumor cells

DISCUSSION

The evaluation of D4-t induced blastogenesis was the first criterion to get an idea whether D4-t could effectively induce blast transformation, and what are the optimal time interval and D4-t concentrations. Blastogenesis is the culmination of several biochemical events, and affects almost every metabolic pathway [23]. The blast cell count in the present investigation shows that D4-t can stimulate lymphoid system of mice *in vivo* and cause blast differentiation of lymphocytes. As it was shown previously by others, ConA seems to be the best stimulating agent of the lymphocytes *in vivo*, with maximal effect at 48 h [18]. In comparison with ConA, and different examined D4-t doses (see Fig. 2, *a–d*), we found that 5 mg/kg of D4-t showed greater degree of stimulation of lymphocytes, as documented by high number of healthy blast cells without any sign of vacuolation or exhaustion. The optimal D4-t dose of 5 mg/kg was confirmed by duplicate screening experiments.

The DNA synthesis assay is also widely used for evaluation of lymphocytes activation *in vivo* [24]. The rate of DNA synthesis by the SP lymphocytes has been followed as the pattern of blastogenesis in current study. By conventional criteria the difference was considered to be very statistically significant ($P < 0.001$). It was previously shown that the optimal dose of ConA for the blastogenic responses of the lymphocytes did not correlate with DNA synthesis [18]. But as we have shown, in the case of D4-t the maximum rate of blastogenesis corresponded to the optimal D4-t dose and correlated with the rate of DNA synthesis.

Next, SP and MLN were isolated from the S-180 tumor bearing mice, treated by 5 mg/kg dose of D4-t, and histological sections were studied. The loosely packed cells of SP possibly reflected the gradual transformation of lymphocytes into blast cells. MLN cells are more sensitive to ConA treatment than SP cells, as it was observed on the histological sections of MLN and SP that most of the MLN cells differentiated into blasts by 48 h [21]. In current study the same effect was observed for the optimal dose of D4-t (data not presented). We didn't observe any abnormalities of cells treated by D4-t at its optimal dose.

It was found that significant ($P < 0.01$) inhibition of tumor growth was in full agreement with the Winn assay data's. In Winn assay tumor mass regression allow us to suggest the antitumor efficacy of D4-t without severe toxicity (see Fig. 4).

As D4-t showed 80% inhibition of ^{3}H-thymidine uptake in comparison to Platin10 and ConA it can be concluded that D4-t could inhibit DNA synthesis of S-180 tumor cells (highly significant $P < 0.001$).

In conclusion, the above results indicate promising therapeutic potential of dimethyl tin 4-cyclohexyl thiosemicarbazone (D4-t).

ACKNOWLEDGEMENTS

We sincerely thank the Department of Science & Technology (DST), Women Scientists Scheme (WOS-A), New Delhi, India, (No.SR/WOS-A/CS-07/2004) for financial assistance to Dr. A. Sen. Also we thank to Dr. U. Sanyal, A. Mukherjee and S. Dutta of Dept. of ACDD & Chemotherapy, Chittaranjan National Cancer Institute, Kolkata for their immense help and support in performing all the radioactive assays for the project.

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