Evaluating the Radioprotective Efficacy of Dendrodoine Analog Against the Formation of Dicentric Aberration Frequency in Cultured Human Peripheral Blood Lymphocytes

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Abstract: The present study was aimed to evaluate the protective efficacy of dendrodoine analog (DA), an aminothiazole derivative against the formation of radiation induced dicentric (DC) aberration frequency on cultured human peripheral blood lymphocytes. DA was chemically synthesized and the product thus obtained was purified using column chromatography packed with silica gel using chloroform as the solvent. The purity status of the final product was assessed employing high performance thin layer chromatography (HPTLC). The radioprotective efficacy of DA against the formation of DC aberration frequency was analyzed by pre-incubating human peripheral blood lymphocytes with the optimum concentration of DA, selected from our previous study, followed by exposure to different doses of radiation. The results indicated that there was a dose dependent increase in the formation of DC aberration frequency in the irradiated groups when compared to DA pre-treated groups which modulated the toxic effects of radiation by means of its effective DNA protective and antioxidant property.

Keywords: Aminothiazole derivative, Dendrodoine analog (DA), High performance thin layer chromatography (HPTLC), Dicentric aberration frequency, Antioxidant property.

1. INTRODUCTION

Cells react differently when subjected to radiation which leads to cellular damage, depending upon the dose of radiation and cell type. If the damage is mild and repairable, it will be handled by the cell's survival netwok but if the damage is extensive and irreparable, the cells will undergo apoptosis. Human peripheral lymphocytes represent a cell population which will be predominantly in a DNA pre-synthetic stage of the cell cycle (i.e. the G0 phase). Only 0.2% or less of the peripheral lymphocytes will be in the auto-synthetic cell cycle phase, and these probably come from the pool of large lymphoid cells representing stimulated lymphocytes or immature plasma cells. Cells from this group may give rise to the rare mitoses found occasionally in peripheral blood. Nowell [1] was the first to show that peripheral 'human leukocytes' can be stimulated by phytohaemagglutinin (PHA) to undergo *in vitro* mitoses, while Carstairs [2] showed that 'small lymphocytes' are the target cells for mitogenic initiation by PHA. In fact, human peripheral blood lymphocytes provide a convenient and readily available source of human material and are routinely used experimentally to assess the extent of cytogenetic damage induced by physical and chemical agents. Sax [3] developed his 'breakage first' hypothesis on the origin of X-ray induced chromosomal aberrations, followed by Revell [4] who proposed the alternative exchange hypothesis. In essence, Sax [3] has proposed that the damaged regions of separate chromosomes come into contact after induction of complete breaks and the ends move about and eventually combine to form exchanges. Alternatively, Revell [4] envisaged that the points of damage are not complete severances but are unstable sites which can interact with similar sites to form pairwise exchanges. There is a third possibility, introduced later by Chadwick and Leenhouts [5], of a lesion/non-lesion interaction whereby a damaged site, in the Revell sense, may interact with an undamaged chromosome to form an exchange. Several studies have employed X-rays to study their effects on radiation induced genetic damage [6-8]. Radiation at doses used in therapy depletes cellular alpha-tocopherol in normal cells, thereby increasing their risk of damage; animal studies show that whole-body exposure to X-ray irradiation decreases tissue concentrations of vitamins C and E [9]. The potential of antioxidants to reduce the cellular damage induced by ionizing radiation has been studied in animal models for more than 50 years. A number of dietary antioxidants have been reported to decrease free radical attack on biomolecules [10]. The application of antioxidant radioprotectors to various human exposure situations has not been extensive although it is generally accepted that endogenous antioxidants, such as cellular non-protein thiols and antioxidant enzymes, provide some degree of protection [11]. These antioxidants exhibit their radioprotective ability

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by either delaying or inhibiting cellular damage mainly through their free radical scavenging property [12]. These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. A wide range of antioxidant phytochemicals, including flavonoids, polyphenols, carotenoids, and organosulfur compounds, are antioxidants and are radioprotective in experimental systems [11, 13].

The marine environment, covering 70% of the earth's surface and 95% of its tropical biosphere represents 34 of the 36 phyla of life and provides a fascinating variety of biodiversity exceeding that of the terrestrial environment. Partially responsible for the unique secondary metabolism of marine life are the ecological pressures in the marine ecosystem including significant competition for space, and a high level of symbiosis between different species [14]. The secondary metabolites of marine organisms possess a number of structural differences due to their biogenetic origin. A variety of marine sources including sponges, tunicates, red algae, acorn worms and symbiotic bacteria have been shown to generate indole alkaloids, which represent the largest number and most complicated of the marine alkaloids (1/4 of total alkaloids) [15]. Marine metabolites often possess complexities such as halogen substituents. Their structure elucidation, chemical modification, stereochemistry, synthesis and pharmacology have received a great deal of interdisciplinary attention from areas of research other than chemistry and include pharmacology, physiology and medicine. Thus compounds isolated or synthesized from marine organisms can be used to develop nutraceuticals with a medical-health benefit, including the prevention and treatment of disease.

Dendrodoine is a marine alkaloid extracted from the tunicate *Dendrodoa grossularia* [16]. It possesses a 1,2,4 - thiadiazole unit, quite uncommon either in terrestrial or among marine natural products. Dendrodoine which belongs to the indole class of marine alkaloids has been reported to be cytotoxic to lymphoma cells L1210 in culture [16]. Grossularine 1 and 2, isolated from the tunicate *Dendrodoa grossularia*, possess cytotoxic properties, against L-1210 (ID_{50} 6 and 4 µg/mL, respectively), WiDr (colon) and MCF7 (breast) (both are < 0.01 µg /mL) [17], and also appear to act as a mono intercalating agent of DNA. Though the synthesis of dendrodoine has been reported [18], further studies on it or its analog have not been reported. It was noted that the substitution of a thiazole ring in place of the thiadiazole ring in Fig. (**1**) would provide additional opportunities for introducing structural diversity. Thiazole moiety is present in a variety of natural and synthetic biomolecules. Aminothiazoles have been reported to possess a wide range of biological activities [19, 20]. The synthesis of complex natural products has been challenging and has led to the discovery of many novel reactions. The synthesis of several compounds has permitted the preparation of a large number of designated analogs. These studies have analyzed desirable modifications, which inevitably lead to the development of more suitable bioactive compounds. High performance thin layer chromatography (HPTLC) is a powerful analytical technique [21] due to its merits of reliability, simplicity, reproducibility and speed. Thus the present study was undertaken initially to investigate the purity profile of the synthesized compound and then to analyze its DNA protecting ability against radiation induced damage on cultured human peripheral blood lymphocytes by performing dicentric (DC) aberration assay.

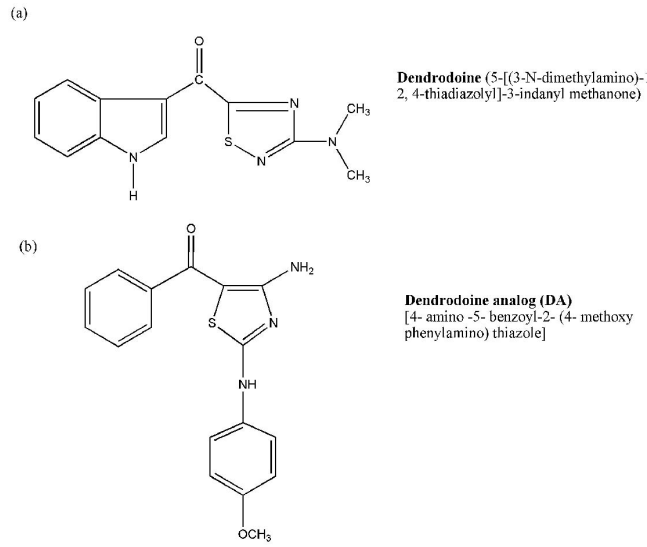

Fig. (1). Molecular structure of dendrodoine and dendrodoine analog (DA).

2. MATERIALS AND METHODS

2.1. Chemicals

Heat inactivated fetal calf serum (FCS), colchicine, histopaque-1077, chloroform (HPLC grade), methanol (HPLC grade) and HPTLC plates silica gel 60 F 254 were obtained from E. Merck, Germany. Other chemicals like penicillin, streptomycin and L-glutamine were purchased from Himedia, Mumbai. Phytohemagglutinin M (PHA-M) was purchased from GIBCO-BRL, USA. All other chemicals and solvents were of analytical grade and were obtained from S.D. Fine chemicals, Mumbai.

2.2. Synthesis of Dendrodoine Analog (DA) and Analyzing its Purity Profile Using High Performance Thin Layer Chromatography (HPTLC)

DA was chemically synthesized in the lab as described by Rajasekaran *et al*., [22] and the product thus obtained was purified using column chromatography packed with silica gel using chloroform as the solvent. The purity check of the final product was confirmed using high performance thin layer chromatography (HPTLC). For this, 20µl of the synthesized compound dissolved in methanol was applied onto HPTLC silica gel plates using CAMAG Linomat 5 instrument. Chloroform: Methanol (9:1) was used as the solvent system to develop the plate.

2.3. HPTLC Operating Conditions

Before operating HPTLC, the pressure in the nitrogen cylinder was ensured to be 4-6 bars. The application position (Y) was fixed at 10.0 mm and the position of the first track (X) was fixed at 15.0 mm. The slit dimension was

fixed as 6.00 x 0.30 mm, Micro. The band length was fixed as 8.0 mm and the applied band was then analyzed using CAMAG Scanner and the resulting peak was detected at 380 nm [23].

2.4. Irradiation Protocol

6 MV X-ray radiation source from the dual energy linear accelerator unit (Dr. Kamakshi Memorial hospital, Chennai, Tamil Nadu, India) was used for the irradiation purpose. Lymphocytes (with or without DA pre-treatment) were exposed to different doses of radiation, depending upon the requirement of the present study.

2.5. Preparation of Dendrodoine Analog (DA) and Isolation of Lymphocytes

DA (1 mg/ml) was dissolved in 0.2% dimethyl sulfoxide (DMSO) (w/v) and kept as stock solution. The stock solution was then diluted with sterile distilled water (milli-Q water) to arrive at the final effective concentration (6 µg/ml) selected from our previous study [24]. Blood samples were aseptically collected in heparinized sterile glass tubes from the median cubital vein of nonsmoking healthy individuals (22-25 years). Written consent was obtained from each one of them. Lymphocytes were isolated from blood using Ficoll-histopaque solution and cultured as described previously by Boyum (1968) [25]. PHA-M (0.2 mL) was added to the culture to initiate cell division. Cells were incubated at 37 $^{\circ}$C in a humidified 5% CO_2 atmosphere. Typically, each culture consisted of an initial density of 1×10^6 cells in 2 ml culture medium.

2.6. Culture set up for investigating the DNA protecting ability of DA by performing dicentric (DC) aberration assay

Sham control	Culture received 0.2% DMSO as vehicle.
DA control	DA (6 µg/ml) pre-treated lymphocytes.
Radiation control	The cultured lymphocytes were exposed to different (1, 2, 3 and 4 Gy) doses of radiation.
DA + radiation	The culture of this group was treated with DA (6 µg/ml) prior to exposure of different (1, 2, 3 and 4 Gy) doses of radiation.

2.7. Dicentric (DC) Aberration Assay and Scoring

To 0.5 ml of the lymphocyte culture (treated/untreated), 5ml culture medium (RPMI-1640) supplemented with $NaHCO_3$ (7.5%, w/v), 20% fetal calf serum, 200 mM L-glutamine, penicillin 100 units/ml and streptomycin 100 µg/ml were added. About 0.2 mL of PHA-M was added to the culture set up to initiate cell division. Colchicine was added at 67 h to block the cells at metaphase stage. The cells were then harvested at 72 h, and were given hypotonic treatment for 10 min and transferred to a pre-cooled slide. The slides were stained with 10% Giemsa, mounted with DPX and examined under oil immersion to score DC aberration frequency [26]. The data are presented as the number of DC/100 cells.

2.8. Statistical Analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by using statistical package of social science (SPSS) version 10.0 for windows. The values are given as mean ± SD of six experiments in each group. P values ≤ 0.05 were considered as level of significance.

3. RESULTS AND DISCUSSION

3.1. Checking the Purity Profile of DA Using HPTLC

The structure of dendrodoine and dendrodoine analog (DA) is shown in Fig. (**1**). Since HPTLC is a powerful analytical technique, it was used to check the purity profile of the synthesized compound DA. This method described utilizes HPTLC silica gel 60 F 254 plates as the stationary phase. Different mobile phases containing different ratios of toluene, methanol, chloroform, acetone and acetonitrile were tested in order to obtain high resolution and reproducible peaks. Finally, the mobile phase chloroform: methanol (9:1, v/v) was selected as more appropriate for obtaining well defined and resolved peaks of DA. The % area covered by peak 1 **(**Fig. **2**), indicative of the compound DA was found to be 98.11 and the area covered by minor impurities was found to be 1.89. Thus the data obtained indicated the purity of DA to be greater than 98%. The Rf values of the peak corresponding to DA is represented in Table **1**. The molecular weight of the purified compound was found to be 325.38 g.

3.2. Dicentric Aberration Assay

Previous work in many laboratories have demonstrated that chromosomal instability can be induced in a variety of mammalian cells by ionizing radiation of different radiation quality [27-31]. Chromosomal instability syndromes are also associated with an increased cancer risk [32] and there are reports stating that an increased rate of chromosomal aberrations indicated an increased risk for secondary cancer [33]. Thus after *in vitro* irradiation of cells, chromosomal aberrations [34] and repair of radiation-induced DNA damage (e.g., single- and double-strand breaks) [35] have been measured and the data obtained has been compared with different types of clinical side effects which might serve as a suitable approach to predict clinical radiation reactions. The frequency of DC aberration formation in human lymphocytes induced by different doses of radiation and in combination with DA is shown in Fig. (**3**). A dose dependent increase in the formation of total DC aberration frequencies was observed in the X-ray irradiated (1, 2, 3 and 4 Gy) groups when compared to the effective concentration of DA (6 µg/ml) pre-treated group. DA pre-treatment prior to the exposure of different doses of radiations (1, 2, 3 and 4 Gy) showed a significant decrease in the formation of DC aberration frequencies and the protection offered by DA was approximately in the range of 80-85%, which is the biological index for the detection of inhibiting potential of DA on cellular toxicity induced by X-ray radiation. Moreover, DA alone treated group did not induce any DC aberration frequencies in the cultured lymphocytes when compared to sham control group. In addition, our previous study also indicated that enhanced levels of lipid

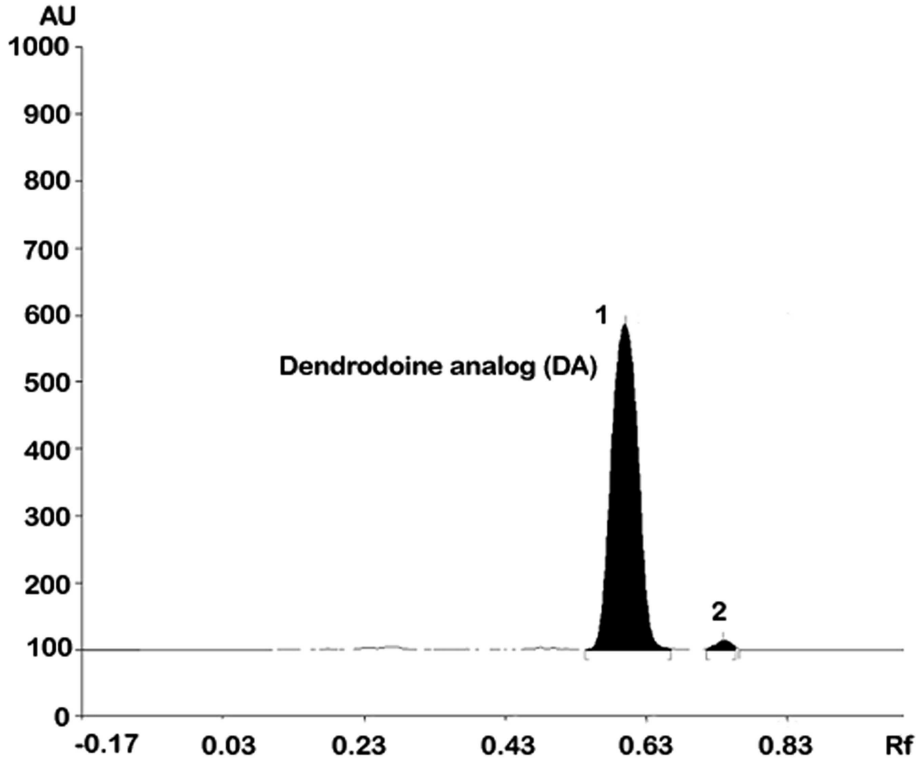

Fig. (2). Peak showing the purity profile of DA as analyzed using HPTLC.

Table 1. Purity Profile Data of Dendrodoine Analog (DA) as Analyzed by HPTLC

Peak	Start Rf	Start Height	Max Rf	Max Height	Max (%)	End Rf	End Height	Area	Area (%)
1	0.55	0.8	0.60	487.8	97.01	0.67	2.3	12727.1	98.11
2	0.72	2.7	0.74	15.0	2.99	0.76	5.3	244.9	1.89

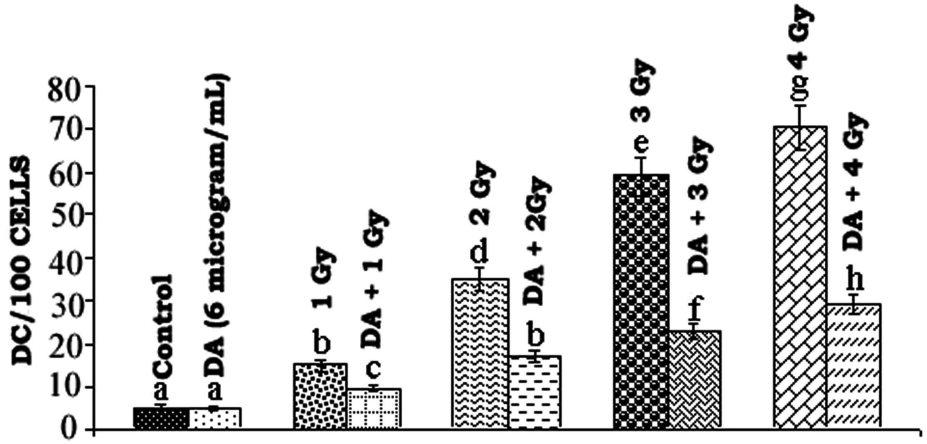

Fig. (3). Inhibitory effect of DA on the formation of DC aberration frequencies induced by different doses of radiation. Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at $P \leq 0.05$.

peroxidation induced by radiation are accompanied by a decrease in the activities of SOD, CAT and GPx in cultured human peripheral blood lymphocytes. However, treatment of lymphocytes with the effective concentration of DA prior to radiation exposure increased the antioxidant status and decreased the level of TBARS [24].

The exact mechanism by which DA develops the anti-clastogenic potential is still unknown. But this may be due to the effective antioxidant potential of DA. As seen in Fig. (**1**), DA was found to possess antioxidant properties due to the presence of amino group at C2, C4 positions and a keto group at C5 position, which accept *electrons* from free

radicals and neutralize them [36]. Previous reports have shown the *in vivo* antioxidant activity of 4-aminothiazoles and have also indicated that aminothiazoles possess antioxidant activity and inhibit lipid peroxidation [20]. In our previous study, we have also reported the antioxidant potential of DA and its protective effect against H_2O_2-induced oxidative damage on pBR322 DNA and RBC cellular membrane [37]. DA has also been reported to decrease the DNA damage by effectively scavenging the free radicals [38]. Moreover DA, besides its antioxidant property, may also form adduct with DNA or other macromolecules thereby preventing any loss of DNA activity. The DA- DNA complex may also be stable, preventing the complexed DNA from undergoing any mutation, resulting in decreased damage to DNA. Further studies are in progress to investigate the *in vivo* radioprotective efficacy of DA.

4. CONCLUSION

The result of the present study indicated that the purity of the synthesized compound DA was confirmed to be greater than 98% by HPTLC. The results on the DNA protecting ability of DA against radiation induced genetic damage on cultured human peripheral blood lymphocytes indicated that DA offered protection to DNA by effectively decreasing the formation of dicentric aberration frequency. Thus the purified compound DA can be used in the field of nutraceutical research due to its valuable antioxidant property with potential applications in health.

CONFLICT OF INTEREST

The authors display no conflict of interest.

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