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Research Article

1α, 25-Dihydroxyvitamin D_3 inhibits hepatic chromosomal aberrations, DNA strand breaks and specific DNA adducts during rat hepatocarcinogenesis

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Abstract. The possible promoting effect of streptozotocin (STZ; 65 mg/kg body weight, intraperitoneal)-induced diabetes during 2-acetylaminofluorene (2-AAF; 0.04 % in basal diet)-initiated hepatocarcinogenesis and modulatory effect of 1α,25-dihydroxyvitamin D_3 (VD_3; 0.3 µg/0.1 ml in propylene glycol, per os) were investigated by monitoring chromosomal aberrations (CAs), DNA strand breaks and specific DNA adducts in rat liver. VD_3 treatment (twice a week) was started 4 weeks before the 2-AAF regimen and continued throughout the study. Aberrant metaphase chromosomes were counted from the regenerating hepatocytes 15, 30 or 45 weeks after STZ injection, while DNA strand break and adduct assays were performed 45 days post-STZ treatment. Dietary exposure to 2-AAF elicited a substantial increase in CAs and elevated the extent of DNA strand breaks and formation of *N*-(deoxyguanosin-8-yl)-2-aminofluorene. A promoting effect of STZ was evident from CAs coupled with DNA strand break analysis. VD_3 treatment substantially reducted 2-AAF+STZ-induced CAs as well as DNA strand breaks and adducts. Thus, VD_3 appears to be effective in suppressing liver-specific early chromosomal as well as DNA damage during the process of rat hepatocarcinogenesis initiated with 2-AAF and promoted by STZ contributing to its promise as a cancer chemotherapeutic agent.

Key words. 2-Acetylaminofluorene; streptozotocin; vitamin D_3; chromosomal aberration; DNA strand break; DNA adduct; rat liver.

Introduction

Epidemiological studies have suggested that supplementation of natural antioxidants may retard or halt oxidative damage leading to disease progression. Potential antioxidant therapy includes natural antioxidant enzymes and vitamins or synthetic agents with antioxidant activity [1]. The biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (VD_3), has been introduced into cancer research with the discovery of large numbers of receptors for VD_3 in at least 60% of all cancer cell lines [2]. VD_3 is an important regulator of cellular growth, differentiation and death [3]. The pancreas is the first non-classical target tissue known to possess the receptor for VD_3, and VD_3 has been reported to induce pancreatic insulin secretion [4]. Earlier studies have established VD_3 as a potent antiproliferative and differentiation- and apoptosis-inducing agent [5, 6]. Treatment of xenografted liver tumour-bearing nude mice with VD_3 retards tumour growth without the development of hypercalcaemia [7]. A protective

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role of VD_3 in chemically induced rat colon carcinogenesis has also been reported [8]. VD_3 possesses antitumour activity against human promyelocytic leukaemia cells [9]. It also inhibits the growth of human liver [7], breast [10] and prostate [11] cancer cell lines in vitro. Experimental studies have shown that diabetes induced by streptozotocin (STZ), a selective pancreatic β-cell toxin and mutagen [12], stimulates the growth of tumours in adult rats [13]. Furthermore, recent reports show that diabetes is associated with an increased risk for several human cancers, notably cancers of the pancreas [14], liver [15], breast [16], colon [17], endometrium [16, 18] and kidney [19].

Accumulating evidence suggests that genomic instability may provide the driving force behind the genetic plasticity characteristic of cancer cells [20] resulting in gene mutation, gene amplification, chromosomal destabilization and cellular transformation [21]. Substantial effort in studying cancer progression has focused on characterizing specific treatments capable of inducing the same endpoints observed in cancer cells, and developing systems capable of accurately measuring the resultant changes [22]. Cytogenetic investigations have demonstrated that a variety of haemopoietic malignancies are characterized by a specific chromosomal abnormality. Recently, the molecular basis of some of these characteristic karyotypical changes has been investigated and structural changes in chromosomes have been demonstrated to alter the expression of specific genes located at breakpoints [22]. The importance of tumour-suppressor genes has been demonstrated, where gain or loss of a part of or an entire chromosome might result in the expression of malignancy.

DNA is the most critical cellular target when considering the lethal carcinogenic and mutagenic effects of drugs, radiation and environmental chemicals [23]. Cellular exposure to ionizing radiation and environmental chemicals results in a variety of directly and indirectly induced DNA lesions including DNA damage caused by base alterations, disruption of the sugar-phosphate backbone, DNA-DNA as well as and DNA-protein cross-links, single-strand breaks and double-strand breaks [24].

Information on the effect of VD_3 in alleviating chemical carcinogenesis or chemically induced diabetes in animal model is meagre, particularly at cytogenetic and molecular levels. However, a recent study from our laboratory [25] demonstrated an inhibitory role of concomitant treatment with micronutrient vanadium and VD_3 on hepatic chromosomal aberrations (CAs) as well as DNA strand breaks during rat liver carcinogenesis induced by diethylnitrosamine (DEN), a potent hepatocarcinogen [26]. VD_3 has also been reported to inhibit chromosomal damage during the growth of Dalton's lymphoma, a transplantable tumour, in mice [27]. Very recently, we documented that the STZ-induced diabetic condition can promote DEN-initiated hepatocarcinogenesis in rats [28] akin to an established promoter, phenobarbital [29] and VD_3 has the potential to suppress rat liver carcinogenesis induced by a combined regimen of DEN and STZ [28]. In the present study, attempts were made to investigate whether the STZ-induced diabetic condition in rats could promote the process of rat hepatocarcinogenesis initiated by 2-acetylaminofluorene (2-AAF), another hepatocarcinogen [26], through analysis of CAs, DNA strand breaks and specific DNA-carcinogen bound adducts in rat liver. We also wanted to elucidate the efficacy of the antioxidant VD_3 in challenging the action of 2-AAF-initiated and diabetes-promoted hepatocarcinogenesis by monitoring these specific molecular markers in the liver to unravel the underlying mechanisms of action of this antitumour agent.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 80–100 g at the beginning of the experiment were obtained from the Indian Institute of Chemical Biology (Calcutta, India). They were kept in wire-mesh metal cages (five rats/cage) at a constant temperature of 22 ± 1°C, relative humidity 50–60% with a 12:12 h controlled light and dark rhythm. The rats were provided with food and water ad libitum. The recommendations of the National Institute of Health, USA for the care and use of laboratory animals were strictly followed in this study.

Experimental regimen

Rats were divided into eight groups (A–H) with 20 rats in each, as illustrated in figure 1. Group B, E, F and H rats were treated with VD_3 (Sigma, St. Louis, MO, USA) at 0.3 µg/ 0.1 ml propylene glycol per os twice a week for the entire period of the experiment with the treatment starting at week 0. From week 4, rats from groups D, E, G and H were fed 0.04% 2-AAF (Sigma) in the basal diet from Monday to Friday through to the end of the experiment. Two weeks after the start of 2-AAF feeding (i.e. week 6), group C, F, G and H rats were intraperitoneally (i.p.) injected with a single dose of STZ (Sigma; 65 mg/kg body weight in 0.05 M citrate buffer, pH 4.5). Normal control (group A) rats received only the vehicles for VD_3 and STZ as above. Groups B, C and D served as VD_3, STZ and 2-AAF controls, respectively. The analysis of CAs was performed from the regenerating hepatocytes at three different timepoints, 15, 30 and 45 days after STZ injection at week 6, following 70% partial hepatectomy 72 h prior to sacrifice under proper ether anaesthesia. For DNA strand break study and specific DNA adduct analysis, hepatic DNA from all the above groups was isolated 18–20 h after the last 2-AAF feeding at day 45.

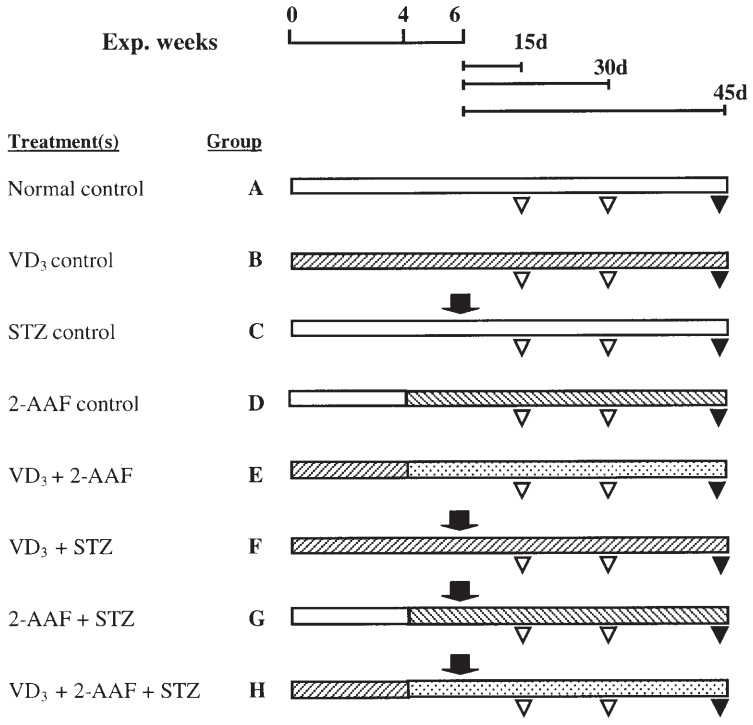

Figure 1. The basic experimental regimen. 1α,25-dihydroxyvitamin D_3 (VD_3) treatment (0.3 µg/0.1 ml propylene glycol) *per os* twice a week. 2-Acetylaminofluorene (2-AAF) feeding (0.04% in basal diet) on 5 consecutive days per week. Treatment with both VD_3 and 2-AAF as above. A single intraperitoneal injection of streptozotocin (STZ; 65 mg/kg body weight). ▽▼ Rats were sacrificed 15, 30 and 45 days after STZ injection at week 6 prior to partial hepatectomy at least 72 h before sacrifice for processing the liver tissue to detect chromosomal anomalies. ▼ Hepatic DNA was isolated 45 days after STZ injection at week 6 for DNA strand break and adduct analysis.

Chromosome preparation from regenerating hepatocytes

Pretreatment was performed by injecting (i.p.) colchicine in 0.9% sodium chloride at 2 mg/kg body weight 3 h before killing. Hepatocytes were isolated following the procedure of Horiuchi et al. [30] that involved incubation of finely minced rat liver slices (about 1 mm^3) with 0.05% collagenase type IV (Sigma) solution for 30 min. The supernatant was then carefully removed and 10 ml of Hanks' solution (Ca^{2+} and Mg^{2+} free) was added to the tissue. The hepatocyte suspension was obtained by gently pipetting the tissue up and down and then allowing it to stand for 5 min. The supernatant was then subjected to centrifugation at 3200 g for 5 min. Pellets of isolated hepatocytes were resuspended in 0.075 M KCl and kept at 37 °C for 25 min. The cells were then fixed with a fixative (methanol-glacial acetic acid, 3:1) that was changed three times and chromosome slides were prepared by flame drying. The slides were kept overnight at room temperature and stained with 3% Giemsa (pH 5.9) for 30 min for scoring CAs.

Scoring of CAs

Metaphase cells with one or more types of CA were scored blind from 50 well-spread metaphase plates per rat (i.e. 250 metaphase plates/group, n = 5) and the frequency of CAs was expressed as the percentage of total aberrant metaphase plates and as the average number of total aberrations. Aberrations were classified into three major groups, namely structural, numerical and physiological aberrations, as in our previously published report [31]. Structural aberrations included gaps, breaks, deletions, fragments, centric fusions, rings and translocations (i.e. direct effect on chromosomes). Numerical aberrations were mainly represented by polyploidy and aneuploidy (i.e. direct effect on the spindle apparatus). Physiological aberrations consisted of stickiness, pyknosis, C-mitotic effect, erosions and pulverizations (i.e. lethal effects).

DNA unwinding assay

DNA was isolated from the rat liver by a modification of the published technique [32] as described previously [31]. DNA purity was checked by determining the ratios of absorbance at A_{260}/A_{280} and A_{260}/A_{230}. The purified DNA solution was then stored at –20 °C. The principle of DNA unwinding is that the fluorescent dye ethidium bromide binds selectively to double-stranded DNA (DS DNA) in the presence of single-stranded DNA (SS DNA) when short duplex regions in SS DNA are destabilized by

alkali treatment [33]. The detailed conditions of the DNA unwinding assay were essentially the same as described in our earlier communication [31]. Briefly, the DNA isolated from each experimental and control group was divided equally into three tubes (B, T and P). The contribution to fluorescence by components other than DS DNA (including the free dye) was estimated from a blank sample (B) in which the DNA solution was first highly sonicated and then treated with alkali under conditions that cause complete unwinding of low molecular weight DS DNA [31]. The second sample (T) was used to determine total fluorescence, i.e. fluorescence due to the presence of DS DNA with contaminants. The difference (T – B) provides an estimate of the total amount of DS DNA in the DNA pool. The third sample (P) was subjected to alkali elution to permit partial unwinding of the DNA [31], the degree of unwinding being related to the size of the DNA. The difference between the fluorescence of the sample and that of the blank (P – B) represents an estimate of the amount of DS DNA remaining. The percentage of DS DNA is given by the equation [31]:

$$\text{DS DNA (\%)} = (P - B) \div (T - B) \times 100$$

Estimation of single strand breaks

The distribution of single-strand breaks in the DNA population is assumed to follow a simple Poisson law. Under these circumstances, the average number of single-strand breaks (n) per DNA unit can be estimated from the simple equation [34]:

$$e^{-n} = D/S + D$$

where S = percentage of DNA that remains single-stranded after alkali treatment, D = percentage remaining as duplex DNA. D/S + D represents the fraction (f_0) of the molecules without strand breaks. The values of n corresponding to different DNA solutions isolated from different groups were then estimated.

Analysis of specific DNA adducts

DNA was enzymatically digested to deoxynucleoside 3′-monophosphates according to the procedure of Gupta [35]. The major DNA adduct, *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) was isolated from an aliquot of DNA digest by extraction with 1-butanol, and then isolated adducts were 5′-^{32}P labelled by polynucleotide kinase phosphorylation as described elsewhere [35]. The ^{32}P-labelled digest was subjected to multidirectional polyethyleneimine-cellulose thin-layer chromatography (TLC) [35, 36] for fingerprinting of ^{32}P-adducts. Analysis of total nucleotides was achieved by direct ^{32}P labelling of another aliquot of the DNA digest as above followed by one-dimensional TLC of the labelled digest [35]. DNA adduct concentration was determined from measurement of radioactivity in adduct as well as total nucleotides and the result was expressed as fmol of adducts/μg DNA, considering 1 μg of DNA = 0.3×10^7 fmol nucleotides [37].

Statistical analysis

Data were analysed statistically for differences between the means using Student's t-test. Values of $P < 0.05$ were taken to imply statistical significance.

Results

Effects of VD_3 on CAs in rats treated with 2-AAF and/or STZ

A significant increase ($P < 0.001$) in the mean number of aberrant metaphase chromosomes was observed in the hepatocytes of 2-AAF controls (group D) compared to normal controls (group A) at different timepoints during the study, i.e. 15, 30 and 45 days (table 1). Most of the aberrations were of the structural type followed by numerical or physiological changes. All types of aberration reached their maxima on day 45. While major structural CAs were exemplified by breaks, gaps, fragments, translocations, centric fusions and rings, the major physiological CAs were sticky aneuploids and sticky hyperdiploids. When STZ was administered following 2-AAF treatment (group G), chromosomal damage was very severe, reflecting its promotional activity. The effect of STZ promotion during 2-AAF-induced hepatocarcinogenesis led to a significant increase in the mean number of aberrations on day 30 and 45 ($P < 0.05$ and 0.001, respectively) when compared to corresponding 2-AAF control (group D) rats. Oral supplementation of VD_3 (groups E, F and H) that started 4 weeks prior to 2-AAF-treatment and continued thereafter for the entire period of the study abated the incidence of various CAs in rat liver cells. A significant ($P < 0.001$) decrease in the mean number of aberrations following VD_3 treatment could be observed in 2-AAF control (group E) rats after 30 and 45 days (35.4 and 43.5%, respectively) and in 2-AAF + STZ-treated rats (group H) at the same timepoints (40.8 and 51.6%, respectively) compared to their control, i.e. groups D and G, respectively. In contrast, supplementation of VD_3 in STZ-treated (group F) rats did not reduce statistically the mean aberrations (25.0, 20.0 and 28.6% at days 15, 30 and 45, respectively) when compared to STZ control (group C) rats. Furthermore, VD_3 treatment alone (group B) demonstrated no clastogenic effect when compared to the normal control (group A) throughout the study.

Effects of VD_3 on hepatic DNA strand breaks in rats treated with 2-AAF and/or STZ

As shown in table 2, the percentage of DS DNA in 2-AAF control (group D) rats was 4-fold less ($P < 0.001$) while the percentage of SS DNA was more than 12-fold higher ($P < 0.001$) than in normal controls (group A). The in-

Table 1. Effects of 1α,25-dihydroxyvitamin D_3 (VD_3) on the frequency distribution of chromosomal aberrations (CAs) in hepatic cells (250 plates/group) of rats treated with 2-acetylaminofluorene (2-AAF) and/or streptozotocin (STZ).

Time (days)	Group	Structural CAs (chromatid breaks, fragments, gaps, deletions, centric fusions, rings, translocations)		Numerical CAs (aneuploidy and polyploidy)		Physiological CAs (stickiness pulverization, pyknosis, C-mitotic effect, erosions)		Total aberrations		Decrease (%)
		number	%	number	%	number	%	number	Mean ± SE (n=5)	
15	A	01	0.40	00	0.00	00	0.00	01	0.20 ± 0.45	
	B	01	0.40	00	0.00	00	0.00	01	0.20 ± 0.45	
	C	02	0.80	01	0.40	01	0.40	04	0.80 ± 0.84	
	D	20	8.00	09	3.60	07	2.80	36	7.20 ± 1.30[a]	
	E	15	6.00	06	2.40	05	2.00	26	5.20 ± 0.83	27.8
	F	02	0.80	01	0.40	00	0.00	03	0.60 ± 0.55	25.0
	G	29	11.6	09	3.60	10	4.00	48	9.60 ± 1.14	
	H	22	8.80	06	2.40	05	2.00	33	6.60 ± 1.14	31.3
30	A	01	0.40	01	0.40	00	0.00	02	0.40 ± 0.55	
	B	01	0.40	01	0.40	00	0.00	02	0.40 ± 0.55	
	C	03	1.20	01	0.40	01	0.40	05	1.00 ± 0.71	
	D	90	36.00	50	20.00	38	15.20	178	35.60 ± 1.14[a]	
	E	68	27.20	21	8.40	26	14.00	115	23.00 ± 1.58[b]	35.4
	F	02	0.80	01	0.40	01	0.40	04	0.80 ± 0.45	20.0
	G	104	41.60	50	20.00	47	10.40	201	40.20 ± 1.48[c]	
	H	60	24.00	29	11.60	30	12.00	119	23.80 ± 1.30[e]	40.8
45	A	02	0.80	01	0.40	01	0.40	04	0.80 ± 0.45	
	B	01	0.40	01	0.40	00	0.00	02	0.40 ± 0.55	
	C	04	1.60	02	0.80	01	0.40	07	1.40 ± 0.55	
	D	114	46.00	57	22.80	60	24.00	232	46.40 ± 1.14[a]	
	E	69	27.60	30	12.00	32	12.80	131	26.20 ± 1.92[b]	43.5
	F	03	1.20	01	0.40	01	0.40	05	1.00 ± 0.71	28.6
	G	148	59.20	70	28.00	71	28.40	289	57.80 ± 1.48[d]	
	H	76	30.40	31	12.40	34	13.60	141	28.00 ± 1.00[e]	51.6

Groups: A, Normal control; B, VD_3 control; C, STZ control; D, 2-AAF control; E, VD_3 + 2-AAF; F, VD_3 + STZ; G, 2-AAF + STZ; H, VD_3 + 2-AAF + STZ. [a] $P < 0.001$ compared to group A. [b] $P < 0.001$, [c] $P < 0.05$ and [d] $P < 0.001$ compared to group D. [e] $P < 0.001$ compared to group G.

Table 2. Effect of 1α,25-dihydroxyvitamin D_3 (VD_3) on DNA strand breaks in rat liver after 2-acetylaminofluorene (2-AAF) and/or streptozotocin (STZ) treatment.

Group	Treatment(s)	DS DNA (mean % ± SE) (n = 5)	SS DNA (mean % ± SE) (n = 5)
A	Normal control	93.75 ± 2.06	06.25 ± 2.06
B	VD_3 control	91.99 ± 1.97	08.01 ± 1.97
C	STZ control	83.75 ± 1.71[b]	16.25 ± 1.71[b]
D	2-AAF control	22.67 ± 1.49[a]	77.33 ± 1.49[a]
E	VD_3 + 2-AAF	38.67 ± 1.65[c]	61.33 ± 1.65[c]
F	VD_3 + STZ	88.83 ± 1.32[f]	11.17 ± 1.32[f]
G	2-AAF + STZ	16.78 ± 1.67[d]	83.22 ± 1.67[d]
H	VD_3+ 2-AAF + STZ	42.36 ± 1.42[e]	57.64 ± 1.42[e]

[a] $p < 0.001$ and [b] $p < 0.01$ compared with normal vehicle control (group A).
[c] $p < 0.001$ and [d] $p < 0.05$ compared with 2-AAF control (group D).
[e] $p < 0.001$ compared with 2-AAF + STZ treated rats (group G).
[f] $p < 0.05$ compared with STZ control (group C).

crease in the percentage of SS DNA is taken as a measure of DNA single-stand breaks. This is actually indicative of the direct DNA-damaging potential of the hepatocarcinogen 2-AAF. Interestingly, our data showed that rats treated with 2-AAF + STZ (group G) experienced an additional ($P < 0.05$) DNA damage in terms of an elevated percentage of SS DNA when compared to 2-AAF control (group D) rats, indicating the promotional activity of STZ. Supplementation with VD_3 in different experimental groups (E, F and H) decreased the percentage of SS DNA following 2-AAF and/or STZ treatment compared to corresponding controls (groups D, C and G, respectively). However, a maximum ($P < 0.001$) inhibitory effect of VD_3 was observed in the VD_3 + 2-AAF + STZ group (group H) followed by the VD_3 + 2-AAF group (group E) ($P < 0.001$) when compared to their controls (groups G and D, respectively). In the 2-AAF (group D) and STZ controls (group C), a significant increase ($P < 0.001$) in

Table 3. Effect of 1α,25-dihydroxyvitamin D_3 (VD_3) on the average number of single strand-breaks/DNA in rat liver after 2-acetylaminofluorene (2-AAF) and/or streptozotocin (STZ) treatment.

Group	Treatment(s)	Average (± SE) number of single-strand breaks/ DNA (n = 5)	Decrease %
A	Normal control	0.06 ± 0.01	
B	VD_3 control	0.08 ± 0.01	
C	STZ control	0.17 ± 0.02 [a]	
D	2-AAF control	1.20 ± 0.15 [a]	
E	VD_3 + 2-AAF	0.65 ± 0.02 [b]	45.8
F	VD_3 +STZ	0.11 ± 0.01 [c]	35.3
G	2-AAF + STZ	1.61 ± 0.04 [c]	
H	VD_3 + 2-AAF + STZ	0.76 ± 0.05 [d]	59.0

[a] $P < 0.001$ compared to normal control (group A).
[b] $P < 0.01$ and [c] $P < 0.05$ compared to 2-AAF control (group D).
[d] $P < 0.001$ compared to 2-AAF + STZ (group G).
[e] $P < 0.05$ compared to STZ control (group C).

Table 4. Effect of 1α,25-dihydroxyvitamin D_3 (VD_3) on formation of *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) in rat liver after 2-acetylaminofluorene (2-AAF) and/or streptozotocin (STZ) treatment.

Group	Treatment(s)	dG-C8-AF (fmol/µg DNA) Mean ± SE (n = 5)	Decrease (%)
A	Normal control	ND [a]	
B	VD_3 control	ND	
C	STZ control	ND	
D	2-AAF control	251.2 ± 23.7	
E	VD_3 + 2-AAF	157.3 ± 17.5 [b]	37.4
F	VD_3 + STZ	ND	
G	2-AAF + STZ	270.3 ± 25.1	
H	VD_3 + 2-AAF + STZ	148.2 ± 12.7 [c]	45.1

[a] ND, not detectable.
[b] $P < 0.05$ compared to 2-AAF control (group D).
[c] $P < 0.01$ compared to 2-AAF + STZ (group G).

the average number of single-strand breaks/DNA was observed 18–20 h after 2-AAF feeding on day 45 when compared to the normal control (group A) (table 2). A single i.p. injection of STZ in conjunction with 2-AAF treatment (group G) also elicited a significant increase ($P < 0.05$) in the average number of single-strand breaks/DNA compared to the 2-AAF control (group D). Treatment with VD_3 offered a 45.8% ($P < 0.01$) and 35.3% ($P < 0.05$) decrease in the average number of single-strand breaks/DNA in VD_3 + 2-AAF (group E) and VD_3 + STZ (group F) rats compared to 2-AAF (group D) and STZ control (group C), respectively. However, a maximum effect (59.0% decrease, $P < 0.001$) was found in VD_3 + 2-AAF + STZ (group H) when compared to 2-AAF + STZ (group G).

Effects of VD_3 on hepatic DNA adducts in rats treated with 2-AAF and/or STZ

As shown in table 4, exposure of rats to an 2-AAF-containing diet in various experimental groups (groups D, E, G and H) resulted in the formation of dG-C8-AF adducts in the liver that was not detectable in other groups (groups A, B, C and F). A significant decrease ($P < 0.05$) in the DNA adduct was observed in the liver of rats subjected to concurrent treatment of VD_3 and 2-AAF (group E) compared to the 2-AAF control (group D). Administration of STZ to 2-AAF-treated rats (group G) increased the level of DNA adducts compared with the 2-AAF control (group D), but the result was not statistically significant. However, VD_3 offered a significant decrease ($P < 0.01$) in this specific DNA adduct in the VD_3 + 2-AAF + STZ group (H) compared to the 2-AAF + STZ group (G).

Discussion

CAs are considered to be good somatic markers as they occur with greatest frequency in cells that are highly proliferative [38]. CAs are known to be important somatic mutations and are clearly involved in the origin, progression and diversification of certain cancers [39]. Considerable biochemical, cytogenetic, molecular genetic and immunological evidence indicates that most neoplasms arise from a single altered cell with the progeny of that cell expanding as a neoplastic 'clone' [40]. Such 'clonal evolution' might result from enhanced genetic instability within the tumour cell population, which increases the probability of further genetic alterations and their subsequent selection [41]. Much of the evidence supporting this clonal-evolution concept has been derived from chromosome studies where somatic genetic changes can be readily visualized [42]. Investigation of the early stages of hepatocarcinogenesis has become possible with the use of cell lines derived from rat liver after the exposure of rats to a carcinogen for a limited period of time [43]. A minimal alteration in chromosomal pattern has been found to correlate with an earlier preneoplastic stage, as shown by chromosomal analysis of DEN-induced tumorigenic and non-tumorigenic rat liver cell lines [44].
In this study, we observed an increase in total CAs including structural aberrations in rat hepatocytes at various time intervals following 2-AAF treatment or a combined regimen of 2-AAF and STZ. Similar results were observed during preneoplasia in rat liver cells using another hepatocarcinogen DEN, as previously reported from our [31, 45] an other [46] laboratories. Another striking observation of the present study was VD_3-mediated suppression of all the structural aberrations studied. A high rate of chromosome breakage has been etiologi-

cally associated with the initiation of carcinogenic process. If the breakage lesions are found to be non-random and the breakage loci are those that are linked to tumorigenesis, then the probability of tumorigenesis in the target tissue increases drastically [47]. Translocations, insertions and inversions that affect genes within a limited region of the breakpoint can result in the deregulation of normal cellular genes or the oncogenic chimeric genes. This response may be even more severe in the event of a deletion of a portion of the chromosome resulting in the genomic loss of essential gene(s) such as suppressor genes, leading to genomic instability. In fact, no such translocations consistently observed in solid tumours have been studied in enough detail to define their threat to the genome during the process of carcinogenesis. Moreover, numerical changes and amplifications leading to alterations in the dosage of large blocks of genes and therefore the specific gene(s) responsible for inducing phenotypic changes in the cell are more difficult to identify [22]. The present investigation clearly demonstrates that VD_3 significantly inhibits chromosomal damages, most specifically the structural changes induced by 2-AAF or 2-AAF + STZ. The chromosomal breaks observed in this study appear to have been random and VD_3 action in vivo is a reflection of its direct antioxidant efficacy in inhibiting these random phenomena. We have revently reported that VD_3 can effectively reduce the incidence of CAs and sister chromatic exchanges (SCEs) in mice carrying Dalton's lymphoma in a time-dependent manner [27]. Moreover, VD_3 in combination with the dietary trace element vanadium can effectively reduce hepatic CAs, especially structural aberrations, during DEN-induced rat hepatocarcinogenesis [25].

Carcinogen-induced DNA damage, DNA repair and SCEs are similar major events during the initial stages of carcinogenesis [48]. DNA strand breaks responsible for chromosome damage are generated from DNA base lesions induced by most chemical mutagens. These DNA base lesions are generally repaired by the excision-repair system [49]. In our present study, a substantial decrease in single-strand breaks may provide the possible mechanism of the anticlastogenic potential of VD_3. The beneficial in vivo effect of VD_3 may be exerted through an effect on excision-repair activity. DNA double-strand breaks are generated from mutagen-induced DNA lesions in the S phase of the cell cycle, and are repaired by post-replicational repair in the G_2 phase; that unrepaired double-strand breaks result in breakage-type CAs [50]. The suppression of breakage-type aberrations by VD_3 treatment may be due to its modification of the post-replicational repair process. Two mechanisms are possible: first, by preventing damage to the chromosome through its antioxidant efficacy, and second, by preventing the DNA breaks by acting on the antiproliferative and differentiation-inducing genes. In vitro VD_3 delays the cell cycle and can thus prolong the repair mechanism. The principal block in cell cycle progression in VD_3-treated human T lymphocytes cells occurs in the G_1 phase [51]. The cyclin-dependent kinase inhibitors $p21^{(Waf1)}$ and $p27^{(Kip1)}$ have been reported to be involved in this process. The $p21^{(Waf1)}$ gene contains a VD_3 response element within its promoter region and its expression increases in response to VD_3 [52]. The expression of $p27^{(Kip1)}$ is markedly induced in certain cancer cell types (e.g. myeloid leukemia and prostate cancer cells) after their exposure to VD_3 in vitro [53, 54]. In view of these reports, the inhibitory effect of VD_3 in vivo on chromosomal damage and DNA strand breaks observed here may be due to its effect in switching the G_1–S checkpoint and thereby allowing repair activity.

Covalent binding of a reactive carcinogen or its metabolite(s) with DNA has been postulated to be the primary event in the initiation phase of chemical carcinogenesis. The hepatocarcinogen 2-AAF or its *N*-hydroxyl derivative have been reported to react exclusively with the guanine moiety in DNA producing two acetylated adducts, *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene and 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene and one deacetylated adduct, dG-C8-AF [36, 55, 56]. The major adduct, dG-C8-AF, represents 90–98% of total adducts measured in genomic DNA isolated from rat liver following 2-AAF exposure [57, 58]. Using the ^{32}P-post-labelling assay, we detected a substantial level of dG-C8-AF in hepatic DNA of rats treated with 2-AAF alone or 2-AAF in combination with STZ. The formation of the dG-C8-AF adduct in conjunction with DNA strand breaks following the 2-AAF regimen observed in the present study indicates a positive correlation between these two molecular events, corroborating a previous report [59]. The observed ability of VD_3 to attenuate the formation of specific DNA adducts in liver may indicate its broad-spectrum potential to modulate the kinetics of adduct formation and removal.

In summary, the results of this study provide strong evidence that the antioxidant VD_3 triggers a unique protective effect against the induction of CAs, DNA strand breaks and specific DNA-carcinogen adducts during rat liver carcinogenesis initiated by a potent hepatocarcinogen 2-AAF and promoted by the STZ-induced diabetes condition, underscoring its promise as a cancer chemopreventive agent.

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