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Azidothymidine induces dose dependent increase in micronuclei formation in cultured HeLa cells

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Exposure of HeLa cells to azidothymidine (AZT) resulted in a concentration dependent decline in growth kinetics. 100 μM of AZT completely inhibited the cell growth. The frequency of binucleate and multinucleate cells declined with increasing concentration of AZT and the formation of multinucleate cells was completely inhibited at 20 and 30 h at higher concentrations indicating inhibition of cell division. Similarly, the clonogenicity of cells declined in a concentration dependent manner and 10 μM AZT killed 50% of the cells. Conversely, the frequency of MNBNC (micronucleated binucleate cell) increased in a concentration dependent manner and was significantly higher in the AZT treated group than the non-drug treated control group. The relationship between concentrations of AZT and micronuclei-induction was linear for all the post-treatment time periods studied. The biological response was also determined by plotting the surviving fraction of cells on the X-axis and the number of micronuclei on the Y-axis. A close and inverse correlation between the surviving fraction and micronuclei formation was observed and the data could be fitted on to a linear quadratic model.

1. Introduction

Azidothymidine (Zidovudine, 3′-azido-3′-deoxythymidine, AZT) is a thymidine analogue first synthesized in the mid 1960s [1], in which the 3′ hydroxyl of the deoxyribose moiety has been replaced by an azido group. Azidothymidine was evaluated as a potential anticancer agent [2, 3]. However, it was found to be more effective as an antiviral agent and has been reported to inhibit replication of Friend leukemia virus [4]. Azidothymidine has been reported to inhibit the infectivity *in vitro* and the cytopathic effects of human immunodeficiency virus type I (HIV-1), and all retroviruses, whether human, avian, murine, feline [5] or simian, [6, 7]. It has also been found to inhibit the replication of human hepatitis B virus [8, 9] and human lymphotrophic virus type 1 (HTLV-1), however, it has been reported to be less effective against HIV-2 virus. Azidothymidine has been reported to induce clinical and immunological improvements in acquired immunodeficiency syndrome (AIDS) patients [10].

Azidothymidine has also been tested for activity against non-retroviruses. The drug inhibited Epstein-Barr virus in concentrations of 1.4 to 2.7 μg/ml, but not herpes simplex or varicella-zoster virus. Many Enterobacteriaceae and *Giardia lamblia* were also inhibited [11]. Azidothymidine has been reported to be effective against Rauschar murine leukemia virus and feline leukemia virus *in vivo* [12].

Azidothymidine is well known in clinical practice for its application in the treatment of AIDS and AIDS-related complex [13, 14], where usually 500–1500 mg/day [15] of the drug is orally administered. Recently, it has been discovered to improve the effect of chemotherapeutic drugs. However, reports regarding its use in non-infected systems are scanty. Therefore, the effect of different concentrations of azidothymidine on cell survival, micronuclei formation and correlation between micronuclei and cell survival in cultured HeLa cells was studied.

2. Investigations and results

The results are expressed as mean ± SEM (standard error of the mean) in Tables 1 and 2.

2.1. Evaluation of optimum duration of drug exposure; Pratt Willis assay

The highest number of viable cells was observed in the non-drug treated control group. The treatment of HeLa cells with various concentrations of azidothymidine resulted in a concentration and drug exposure duration dependent decline in the number of viable cells. A maximum cell killing effect of AZT was observed when the drug was available to HeLa cells up to 8 h (Fig. 1) and therefore, 8 h duration of drug exposure was selected for further studies.

2.2. Growth kinetics

The number of viable cells increased with the scoring time in the non-drug treated group, and a peak number of viable cells was observed on day 5 post-treatment (Fig. 2). Exposure of HeLa cells to various concentrations of AZT resulted in a concentration dependent decline in the number of viable cells. However, with each scoring time the number of viable cells also increased for each AZT concentration and a peak number of viable cells was scored on day 5 post-treatment, except for 100 μM AZT, where no appreciable change in the number of viable cells could be observed with scoring time (Fig. 2). The number of cells scored in the AZT treated group was significantly lower than of the non-drug treated control.

2.3. Clonogenic assay

Treatment of HeLa cells with various concentrations of AZT resulted in a concentration dependent decline in cell survival (Fig. 3). The surviving fraction of cells declined by 0.26 at a concentration of 0.01 μM, the lowest concentration of AZT used. However, increasing concentration of AZT resulted in a further decline in the surviving fraction and a two fold decline in cell survival was observed at 10 μM AZT, where the surviving fraction was reduced to 50% of the control.

Table 1: Induction of micronuclei by various concentrations of azidothymidine in HeLa cells at different post-treatment time periods

AZT (μM)	Frequency of micronucleated binucleate cells per 1000 ± SEM																		
	Post-treatment scoring time period (hours)																		
	20							30							40						
	A	B	C	D	E	F	mean	A	B	C	D	E	F	mean	A	B	C	D	E	F	mean
One MN																					
0	11	6	12	9	10	9	9.50 ± 0.85	30	35	28	40	31	36	33.33 ± 1.82	14	18	15	15	17	14	15.50 ± 0.67
0.01	12	20	16	13	15	16	15.33 ± 1.14	45	47	54	42	44	49	46.83 ± 1.70[b]	23	26	20	22	23	24	23.00 ± 0.82[a]
0.1	22	26	23	22	20	19	22.00 ± 1.00[b]	54	56	54	53	60	52	54.83 ± 1.17[c]	33	33	31	34	29	42	33.67 ± 1.82[c]
1.0	28	30	38	26	22	25	28.17 ± 2.26[c]	78	80	73	69	79	80	76.50 ± 1.84[c]	42	38	40	41	38	44	40.50 ± 0.96[c]
10	32	39	30	40	29	34	34.00 ± 1.88[c]	114	112	108	113	109	111	110.50 ± 0.76[c]	64	71	68	63	65	62	65.50 ± 1.38[c]
100	48	47	45	46	38	44	44.67 ± 1.45[c]	114	118	116	110	113	116	114.50 ± 1.15[c]	75	74	78	80	77	72	76.00 ± 1.18[c]
r							0.99							0.98							0.98
Two MN																					
0	1	0	0	1	0	0	0.33 ± 0.21	2	0	1	0	2	0	0.83 ± 0.40	1	0	0	1	0	1	0.50 ± 0.22
0.01	4	2	3	6	4	3	3.67 ± 0.56[a]	6	8	5	4	5	4	5.33 ± 0.61[a]	4	6	7	5	3	2	4.50 ± 0.76[a]
0.1	4	5	3	6	4	5	4.50 ± 0.43[a]	9	10	8	7	4	6	7.33 ± 0.88[b]	6	8	5	4	8	4	5.83 ± 0.75[b]
1.0	5	8	3	4	6	8	5.67 ± 0.84[b]	13	16	12	15	11	12	13.17 ± 0.79[c]	5	10	7	4	7	6	6.50 ± 0.85[b]
10	7	5	8	6	10	5	6.83 ± 0.79[c]	15	18	16	19	14	19	16.83 ± 0.87[c]	8	9	7	10	5	8	7.83 ± 0.40[c]
100	8	9	10	6	7	8	8.00 ± 0.58[c]	20	25	21	19	17	18	20.00 ± 1.15[c]	12	11	9	10	14	11	11.17 ± 0.70[c]
r							0.97							0.99							0.96
Multiple MN																					
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.01	1	2	1	0	2	1	1.17 ± 0.31	2	3	1	2	2	3	2.17 ± 0.31	1	0	2	3	1	0	1.17 ± 0.48
0.1	2	1	1	2	2	1	1.50 ± 0.22[a]	2	4	6	2	5	3	3.67 ± 0.67[a]	3	4	3	2	1	2	2.50 ± 0.43
1.0	2	2	1	2	3	2	2.00 ± 0.26[b]	4	6	7	5	3	2	4.50 ± 0.76[b]	3	6	5	4	2	1	3.50 ± 0.76
10	3	2	2	3	2	3	2.50 ± 0.22[c]	5	8	7	6	4	5	5.83 ± 0.60[c]	3	5	9	6	2	3	4.67 ± 1.05[a]
100	4	2	4	4	5	3	3.67 ± 0.42[c]	6	9	8	7	8	5	7.17 ± 0.60[c]	8	7	9	4	2	3	5.50 ± 1.18[b]
r							0.98							0.99							0.998
Total MN																					
0	12	6	12	10	10	9	9.83 ± 0.91	32	35	29	40	33	36	34.17 ± 1.54	15	18	15	16	17	15	16.00 ± 0.52
0.01	17	24	20	19	21	20	20.17 ± 0.94[b]	53	58	60	48	51	56	54.33 ± 1.84[c]	28	32	29	30	27	26	28.67 ± 0.88[b]
0.1	28	32	27	30	26	25	28.00 ± 1.06[c]	65	70	68	62	69	61	65.83 ± 1.54[c]	42	45	39	40	38	48	42.00 ± 1.57[c]
1.0	35	40	42	32	31	35	35.83 ± 1.78[c]	95	102	92	89	93	94	94.17 ± 1.78[c]	50	54	52	49	47	51	50.50 ± 0.99[c]
10	42	46	40	49	41	42	43.33 ± 1.41[c]	130	138	131	138	127	135	133.17 ± 1.85[c]	75	85	84	79	72	73	78.00 ± 2.28[c]
100	60	58	59	56	50	55	56.33 ± 1.47[c]	140	152	145	136	138	139	141.67 ± 2.40[c]	95	92	96	94	93	86	92.67 ± 1.45[c]
r							0.996							0.98							0.99

p < a = 0.05, b = 0.01, c = 0.001 No symbol = non-significant
AZT = azidothymidine

Table 2: Alteration in cell proliferation indices in HeLa cells exposed to various concentrations of azidothymidine at different post-treatment time periods

AZT (μM)	Frequency of nucleated cells per 1000 ± SEM																		
	Post-treatment scoring time period (hours)																		
	20							30							40						
	A	B	C	D	E	F	mean	A	B	C	D	E	F	mean	A	B	C	D	E	F	mean
Mononucleate																					
0	680	665	640	652	656	658	658.50 ± 5.46	260	265	256	268	250	269	261.33 ± 3.03	132	128	125	130	127	131	128.83 ± 1.08
0.01	860	865	864	858	862	859	861.33 ± 1.14[c]	479	478	482	486	475	473	478.83 ± 1.92[c]	258	262	266	264	265	259	262.33 ± 1.33[c]
0.1	910	912	903	908	916	906	909.17 ± 1.87[c]	544	542	540	543	539	545	542.17 ± 0.94[c]	325	334	342	330	336	340	334.50 ± 2.58[c]
1.0	924	935	920	928	918	936	926.83 ± 3.08[c]	600	596	593	585	609	599	597.00 ± 3.25[c]	386	395	392	391	390	395	391.50 ± 1.38[c]
10	958	960	973	961	968	962	963.67 ± 2.32[c]	612	609	606	610	613	605	609.17 ± 1.30[c]	458	433	448	430	462	445	446.00 ± 5.26[c]
100	988	990	981	989	986	985	986.50 ± 1.33[c]	699	702	697	692	699	705	699.00 ± 1.81[c]	482	496	493	490	495	497	492.17 ± 2.27[c]
Binucleate																					
0	312	323	320	318	324	316	318.83 ± 1.83	719	708	728	715	722	720	718.67 ± 2.75	246	256	250	258	252	247	251.50 ± 1.96
0.01	139	132	138	128	145	135	136.17 ± 2.41[c]	510	506	509	515	508	505	508.83 ± 1.44[c]	228	217	223	230	225	218	223.83 ± 1.96[c]
0.1	88	86	96	90	91	89	90.00 ± 1.39[c]	447	446	454	450	448	445	448.33 ± 1.33[c]	198	186	192	190	197	192	192.50 ± 1.82[c]
1.0	75	65	80	81	72	68	73.50 ± 2.62[c]	396	398	405	392	410	397	399.67 ± 2.69[c]	168	159	160	165	162	163	162.83 ± 1.35[c]
10	43	42	45	41	52	44	44.50 ± 1.61[c]	386	388	394	398	382	387	389.17 ± 2.37[c]	138	125	119	136	128	118	127.33 ± 3.42[c]
100	25	30	22	26	22	23	24.67 ± 1.26[c]	300	298	303	295	306	302	300.67 ± 1.58[c]	110	120	116	109	106	113	112.33 ± 2.08[c]
Multinucleate																					
0	6	2	2	4	3	2	3.17 ± 0.65	21	27	16	24	21	27	22.67 ± 1.72	622	616	625	632	622	621	623.00 ± 2.16
0.01	1	3	4	2	4	1	2.50 ± 0.56	11	16	9	15	13	9	12.17 ± 1.22[c]	514	519	512	520	514	510	514.83 ± 1.60[c]
0.1	2	2	1	3	1	2	1.83 ± 0.31	9	12	6	10	11	8	9.33 ± 0.88[c]	477	480	466	486	472	467	474.67 ± 3.18[c]
1.0	1	0	0	1	0	0	0.33 ± 0.21[b]	4	6	2	5	2	6	4.17 ± 0.75[c]	446	446	448	444	456	444	447.33 ± 1.84[c]
10	0	0	0	0	0	0	0[b]	2	3	1	0	2	2	1.67 ± 0.42[c]	404	442	433	435	430	412	426.00 ± 6.00[c]
100	0	0	0	0	0	0	0[b]	1	0	0	0	1	0	0.50 ± 0.22[c]	400	392	396	389	389	409	395.83 ± 3.16[c]

$p < a = 0.05$, $b = 0.01$, $c = 0.001$ No symbol = non-significant
AZT = azidothymidine

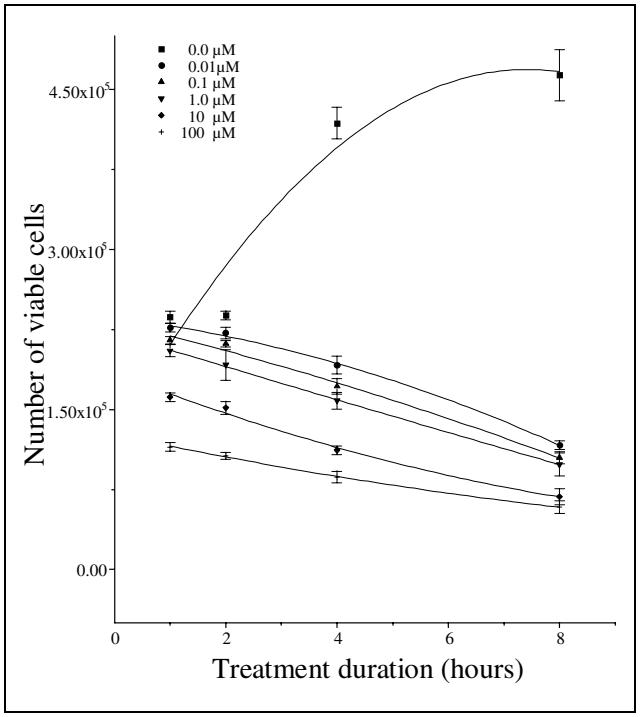

Fig. 1: Effect of different treatment times on the viability of HeLa cells treated with various concentrations of azidothymidine

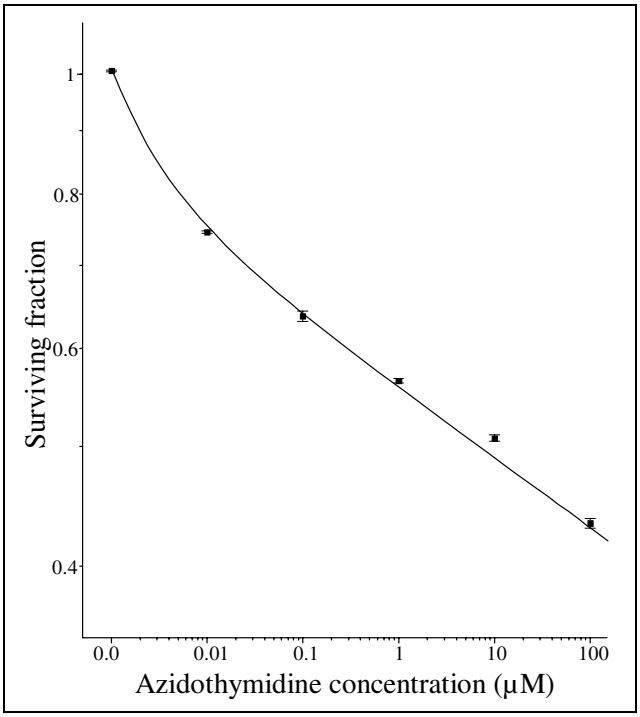

Fig. 3: Effect of various concentrations of azidothymidine on the survival of HeLa cells

2.4. Micronuclei

Exposure of HeLa cells to various concentrations of AZT resulted in a concentration dependent elevation in the frequency of micronucleated binucleate cells (MNBNC) at all the post-treatment time periods (Fig. 4). The frequency of MNBNC was significantly higher at 20, 30 and 40 h post-treatment for all the concentrations of AZT. The frequency of MNBNC increased 2.7 fold after exposure to 1 μM AZT when compared to the non-drug treated controls, while this elevation in MNBNC frequency was approximately 4 fold for 10 and 100 μM AZT, respectively. The distribution of binucleate cells bearing one, two and multiple micronuclei (> two micronuclei in one binucleate cell) is shown in Table 1. The frequency of one MNBNC increased in a concentration dependent manner (Fig. 4b). This increase was significantly higher in those groups treated with 0.01 to 100 μM AZT at 30 and 40 h post-treatment. At 20 h, a non-significant elevation in one MNBNC was observed at 0.01 μM AZT. The frequency of two MNBNC also elevated in a concentration related manner (Fig. 4c) and was significantly higher after treatment with 0.01 to 100 μM AZT at 20, 30 and 40 h post-treatment (Table 1). Treatment of HeLa cells with different concentrations of AZT induced multiple MNBNC in a concentration dependent manner (Fig. 4d). The frequency of multiple MNBNC was significantly elevated at 20 and 30 h after exposure to 0.1 to 100 μM AZT, except that there was a non-significant elevation in the multiple-MNBNC frequency after exposure to 0.01 to 1 μM AZT at 40 h (Table 1).

The frequency of MNBNC increased up to 30 h post-treatment and declined thereafter. However, the frequency of MNBNC was higher at 40 h when compared with 20 h post-treatment. This increase in MNBNC frequency was linear at all the post-treatment time periods studied (Table 1).

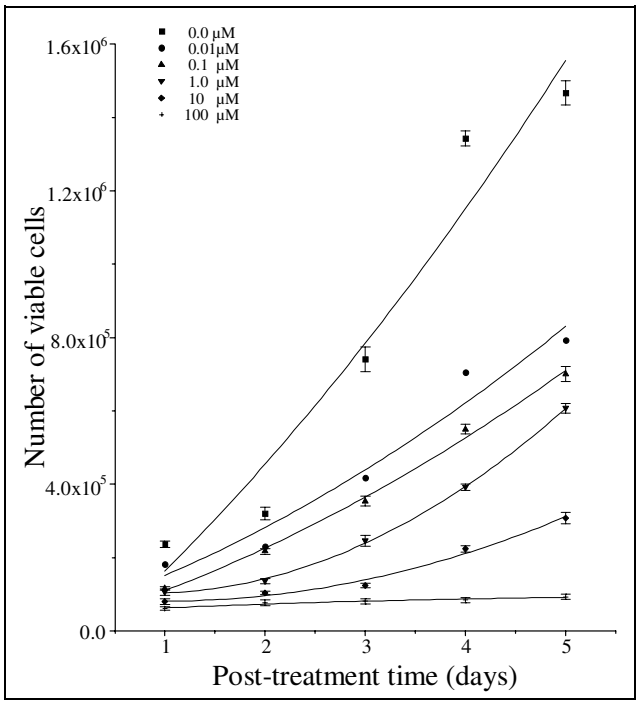

Fig. 2: Alteration in growth kinetics of HeLa cells treated with various concentrations of azidothymidine

2.5. Cell proliferation index

Treatment of HeLa cells with various concentrations of AZT resulted in a dose dependent decline in the frequency of binucleate cells at all the post-treatment time periods (Fig. 5b). This decline in the binucleate cells was significantly higher for all the AZT concentrations at 20, 30 and 40 h post-treatment. In contrast, the frequency of mononucleated cells showed a concentration dependent elevation (Fig. 5a). The frequency of mononucleate cells was significantly higher at all the concentrations of AZT at all the post-treatment time periods when compared with the non-

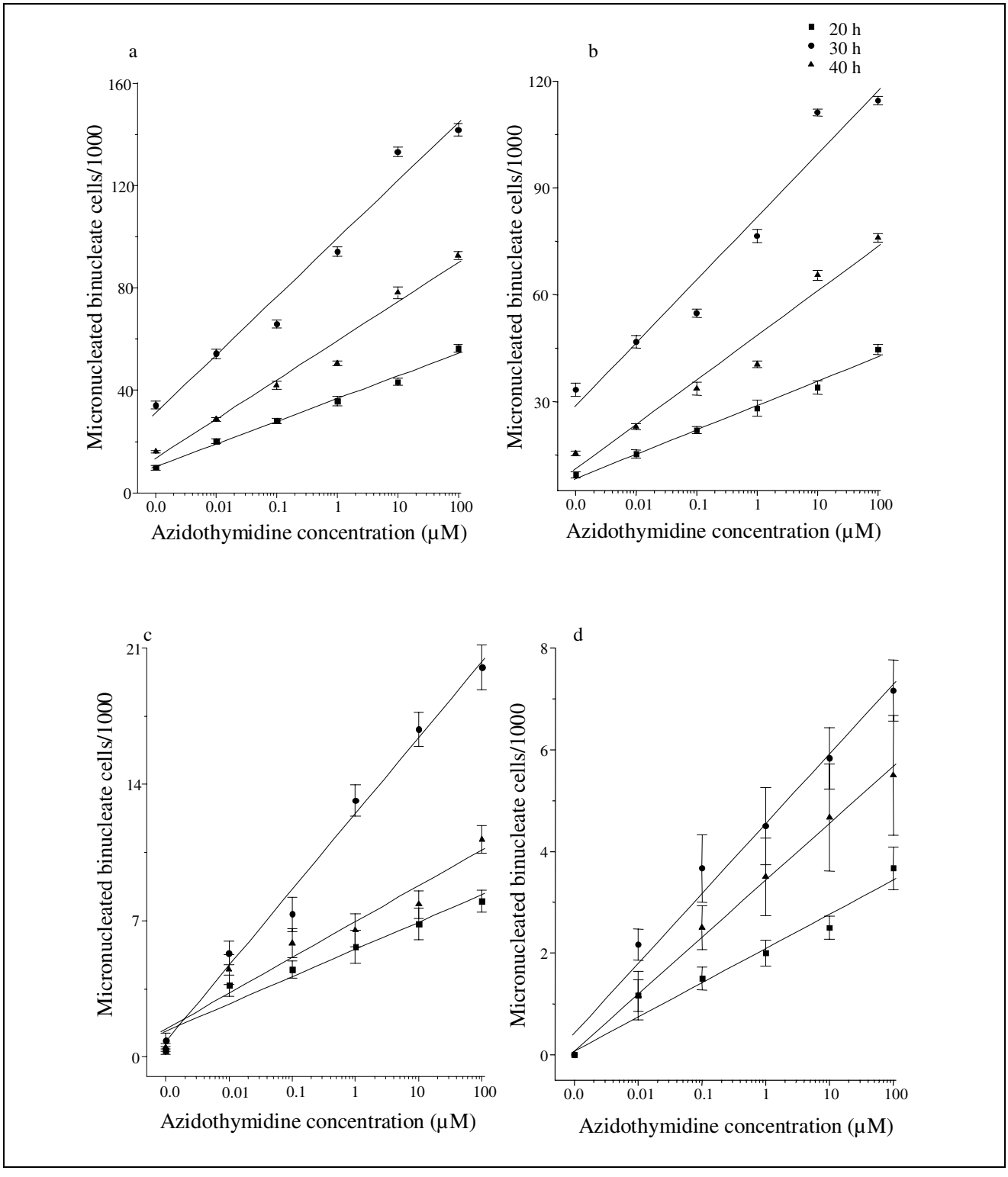

Fig. 4: Effect of various doses of azidothymidine on the micronucleus formation in HeLa cells at different post-treatment time periods. a) total MNBNC; b) one MNBNC; c) two MNBNC; d) multiple MNBNC

drug treated controls. The treatment of HeLa cells with AZT resulted in a concentration dependent decline in the multinucleate cells at all the post-treatment scoring time periods when compared with non-drug treated control (Fig. 5c, d). The multinucleate cells were absent after exposure to 1 to 100 μM at 20 h and 100 μM at 30 h, indicating a block in the cell division. A highest elevation in multinucleate cells was observed at 40 h post-treatment when compared to 20 and 30 h (Fig. 5d).

2.6. Correlation between cell survival and micronuclei induction

The correlation between cell survival and micronuclei induction was determined by plotting the surviving fraction of cells on the X-axis and MNBNC frequency on the Y-axis. The data were fitted to the linear quadratic model ($Y = C + \alpha D + \beta D^2$). The surviving fraction of cells declined with the increase in micronuclei frequency (Fig. 6)

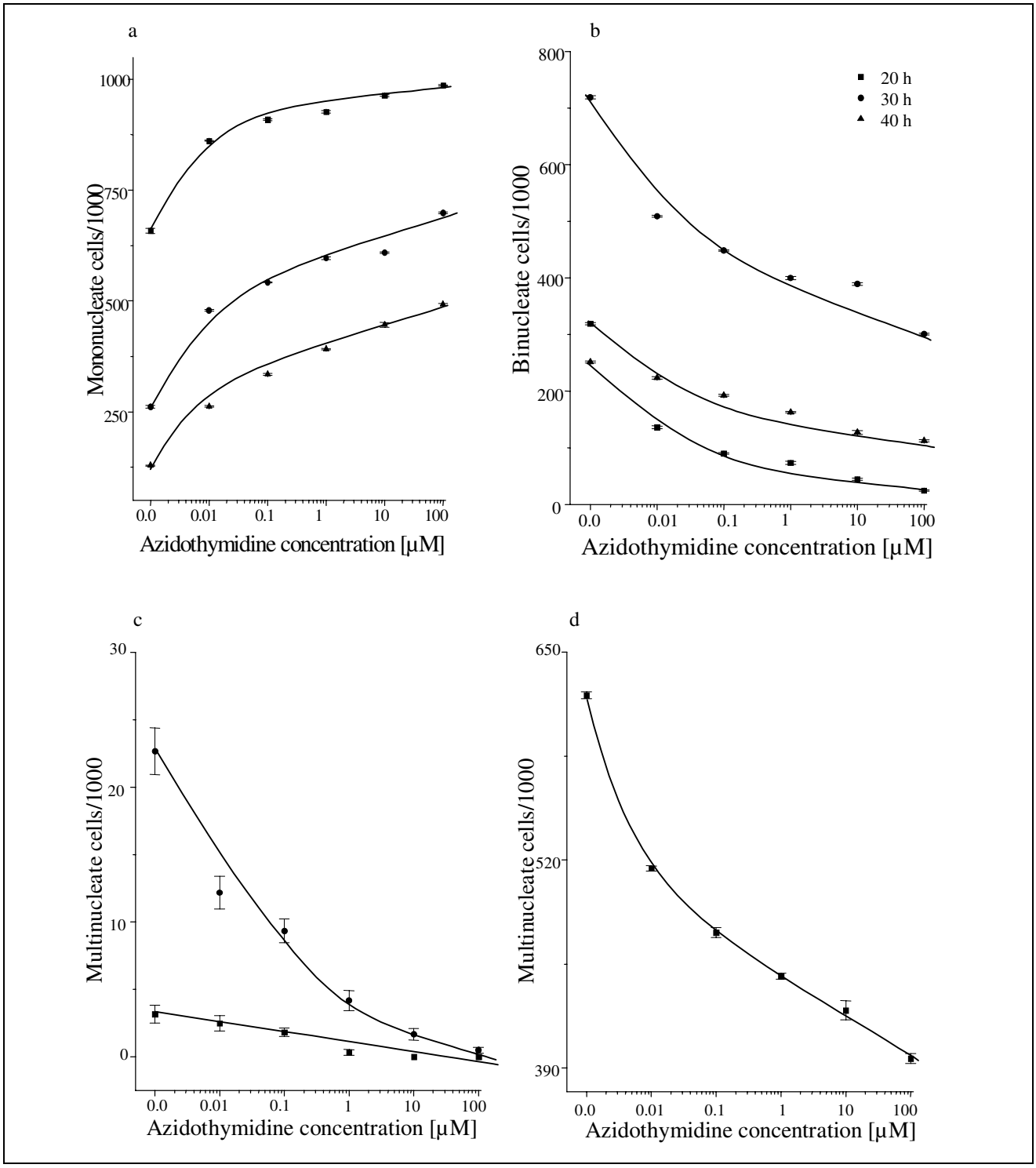

Fig. 5: Alteration in cell proliferation indices of HeLa cells exposed to different concentrations of azidothymidine. a) mononucleate cells; b) binucleate cells; c) and d) multi-nucleate cells

and this relationship between MNBNC and surviving fraction was linear quadratic ($r = 0.999$, 0.98, 0.98 for 20, 30 and 40 h, respectively).

3. Discussion

Azidothymidine is used for the treatment of AIDS and AIDS related disorders in humans. Azidothymidine is a thymidine analogue and apart from killing the virus it may also adversely affect the normal cellular genome of the host. Exposure of HeLa cells to various concentrations of AZT caused a concentration dependent decline in the growth kinetics of HeLa cells and 100 μM AZT inhibited the cell division, as there was no appreciable change, in the number of viable cells at 5 day post-treatment. AZT has been reported to inhibit the proliferation of human bone marrow progenitor cells *in vitro* [16]. Somewhat similar results have been reported in four breast cancer cell lines, T4 cell leukemia cells and a normal breast cell line *in vitro* [17]. The evaluation of cell proliferation indices indicates that binucleate and multinucleate cells declined in a dose dependent manner and the highest decline in

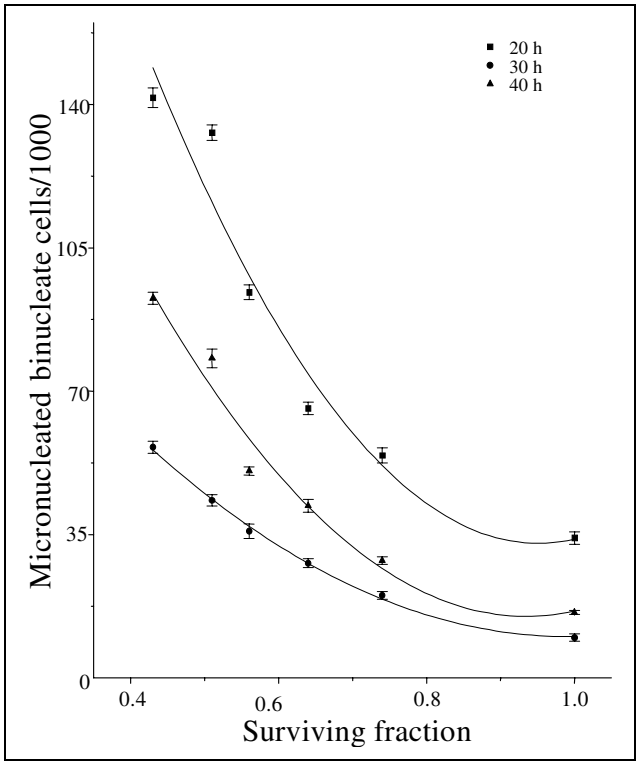

Fig. 6: Correlation between cell survival and micronuclei-induction in HeLa cells treated with various concentrations of azidothymidine

multinucleate cells was observed at 20 and 30 h post-treatment. This shows that AZT has inhibited the progression of cells towards mitosis. A concentration dependent prolongation in the cell doubling time has been reported in HL60 cells [18]. A delay in lymphocyte kinetics has also been observed, where mitotic index and second metaphase declined with increasing dose of AZT [19].

The growth kinetics and cell proliferation studies have been confirmed by the clonogenic assay, where the surviving fraction of the cells declined with the increasing concentration of AZT and 50% of the cells were killed at a concentration of 10 μM. A dose dependent decline in the cell proliferation and cell viability have been observed for human keratinocytes treated with various concentration of AZT *in vitro* [20]. AZT has been found to inhibit the growth of Sp2/0 cells *in vitro* [21]. A 50% cell killing by 10 μM and 25 μM AZT has been observed for human CEM-T cells and several other human and animal cells [22]. AZT has been shown to inhibit ^{3}H thymidine uptake in CEM and cultured human peripheral blood lymphocytes and also to reduce the number of cell generations [23]. AZT has been reported to inhibit the growth of HCT-8 cells with an IC_{50} of 21.5 μM [24]. Similarly, ID_{50} of 70 μM has been reported for human fibroblasts [25]. This variation in LD_{50} dose may be due to the differential metabolism of AZT in different cell lines.

The AZT treatment resulted in a concentration dependent elevation in the frequency of MNBNC in HeLa cells and at all the post-treatment time periods it was significantly higher than that of the non-drug treated controls. A concentration dependent increase in the micronuclei has been reported in human lymphocytes treated with 10 to 500 μg/ml of AZT [26]. AZT treatment has been reported to increase chromosomal aberrations in human T-lymphocytic H9 cells [27]. The elevation in MNBNC frequency was significant even with the lowest dose of 0.01 μM in the present study and the highest dose was far lower than 500 μg/ml used in the above study. A concentration dependent increase in the micronucleated erythrocytes has been reported in the bone marrow of mice administered with 100–1000 mg/kg/day of AZT orally for four weeks. A similar increase in micronuclei has also been reported after 4 or 7 days of 500 mg/kg/day of AZT in rats and mice [28]. Similarly, AZT has been found to increase the frequency of micronuclei in mouse erythrocytes [29, 30]. In the present study a significant increase in MNBNC frequency was observed for all concentrations of AZT at all the time periods. AZT has been reported to induce chromosome breakage in a concentration dependent manner with linear relationship in human lymphocytes and CHO cells [19]. It has also been found to elevate the micronuclei frequency in CD4 cells [31]. AZT has been reported to induce sister chromatid exchanges in human lymphocytes and CHO cells [26]. We have also reported a linear increase in MNBNC frequency in the present study. AZT has been shown to induce chromosomal aberrations at 3 μg/ml in human lymphocytes and higher doses caused cell transformation [32]. AZT has been able to induce BNC with $\geq$ MN in a dose dependent manner indicating its efficacy in causing multiple damage to the cellular genome and induction of complex chromosome aberrations that might have been responsible for cell death.

The biological response was determined by plotting the MNBNC frequency on Y- and surviving fraction on X-axes, respectively. It was observed that by increasing MNBNC frequency the surviving fraction declined, indicating a close correlation between the induction of MNBNC and cell survival. This decline in cell survival may be due to the loss of a significant amount of genome in the form of micronuclei that may be lost with the subsequent cell division causing cell death. The correlation between surviving fraction and micronuclei-induction was linear quadratic. This is in conformation with our earlier studies, where a similar correlation has been reported in V79 and HeLa cells with different drugs [33–38]. The other authors have also reported a close correlation between cell survival and micronuclei formation [39–42], however, they reported a linear relationship. This may be due to variation in cell proliferation kinetics and the use of cells in various phases of cell cycle. In the present study plateau phase cells in G_0/G_1 have been used and the cell survival and micronucleus assays were carried out concurrently from the same stock of cells.

The mechanism of action of AZT is not fully understood. However, AZT is phosphorylated into mono-di- and triphosphates by the enzyme thymidine kinase, thymidylate kinase and cell nucleoside diphosphate kinase, respectively [24]. The generation of intracellular pools of AZTMP may be responsible for the cytotoxic effects of AZT [43]. AZT has been found to be incorporated into the cellular DNA [16] and has been reported to induce damage to the DNA [44]. AZT has recently been reported to induce DNA double-strand cleavage in Sp2/0 cells [21]. The DNA double strand breaks caused by AZT may subsequently be converted into chromosome breaks and finally to micronuclei. The failure of repair of DNA damage induced by AZT may be responsible for the increased micronuclei frequency and the reduction in the cell survival in AZT-treated group, when compared with non-drug treated control.

Our study demonstrates that AZT causes changes in the fidelity of cell genome as evidenced by the induction of MNBNC. The drug not only-induced cells bearing one

MNBNC but also cells with multiple MNBNC, indicating that the drug is able to induce multiple lesions in the DNA of the cell.

4. Experimental

4.1. Drugs and chemicals

An appropriate amount of azidothymidine (AZT, Cipla Ltd., Bangalore, India) was dissolved in sterile double distilled water (DDW) and the drug was diluted to the required concentrations. A constant volume of 50 µl per 5 ml medium was added to individual cell cultures irrespective of the drug concentration. Cytochalasin-B (Sigma cat. No. C-6762) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml, stored at −80 °C and diluted with sterile PBS immediately before use. Cytochalasin-B, MEM, L-glutamine, gentamicin sulfate, fetal calf serum and DMSO were obtained from Sigma Chemical Co., St. Louis, USA.

4.2. Cell line and culture

HeLa cells procured from National Centre for Cell Science, Pune, India, were used throughout this study. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg/ml gentamicin sulfate. Cells were routinely grown in 75 cm^2 flasks with loosened caps, and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

4.3. Evaluation of optimum drug treatment time

The optimum duration for drug exposure was evaluated by the Pratt and Willis test [45]. Usually 3×10^4 cells were seeded in 25 cm^2 petridishes in triplicate for each drug dose. The cells were allowed to grow for 24 h and were treated with 0, 0.01, 0.1, 1, 10 and 100 µM AZT for 1, 2, 4 and 8 h. The drug containing media was replaced with the fresh medium after 1, 2, 4 and 8 h of AZT treatment and the cells were left undisturbed. The cells were dislodged by trypsin EDTA treatment 72 h after the initiation of the cell cultures and the viability of cells was determined using trypan blue dye exclusion.
The optimum period of time for drug exposure was determined depending on the loss of cell viability. A maximum cell kill by various concentrations of AZT was observed for 8 h drug treatment and the further experiments were carried out using this duration for drug treatment.

4.4. Experimental protocol

A fixed number of exponentially growing cells were inoculated into 25 cm^2 culture flasks (Nunc, Rosklide, Denmark) and allowed to attain plateau phase. The plateau phase cells were exposed continuously for 8 h to different concentrations viz. 0, 0.01, 0.1, 1, 10 and 100 µM of azidothymidine. Eight hours after AZT treatment, the drug containing medium was removed and the cells were dislodged from the culture flasks by trypsin EDTA treatment, they were divided into three parts and the following studies were carried out concurrently for each drug concentration.

4.5. Growth kinetics

One part of the cells was used to evaluate growth kinetics, where 10^4 cells were inoculated into 25 cm^2 petridishes in triplicate for each drug concentration. The cells were allowed to grow for 1, 2, 3, 4 and 5 days. The cells from each culture dish were detached at the time periods specified above. The cells were stained with trypan blue and scored under an inverted microscope (Leitz, Germany) using a hemocytometer (American optical Co., USA). The growth kinetics was determined by scoring viable cell numbers for each concentrations of individual dishes. The results of azidothymidine treatments were compared with the non-drug treated controls.

4.6. Clonogenic assay

The second part of the cells was used for clonogenic assay. Clonogenicity of cells was measured using the colony forming assay of Puck and Marcus [46]. 200–300 cells were inoculated in triplicate for each drug concentration in 5 ml medium and allowed to grow for 11 days. The resultant colonies were stained with 1% crystal violet in methanol and clusters containing 50 or more cells were scored as a colony. The plating efficiency for all drug doses was determined and the data are expressed as surviving fraction. The data were fitted on a linear quadratic model $SF = \exp -(\alpha D + \beta D^2)$.

4.7. Micronucleus assay

The third part of the cells was used for micronucleus assay, where 3×10^5 cells were inoculated in triplicate for each drug dose. Briefly, the micronuclei were prepared according to the modified method of Fenech and Morley [47]. The cells were allowed to attach for six hours, after that the cells were treated with 3 µg/ml of cytochalasin-B to inhibit cytokinesis. The cells were left undisturbed and were allowed to grow for another 14, 24 and 34 h depending on the assay time. The cell cultures were terminated at 20, 30 and 40 h post-drug-treatment. The media containing cytochalasin-B were removed and the cells were washed with PBS. Finally, the cells were dislodged by trypsin EDTA treatment, centrifuged, subjected to mild hypotonic treatment (0.7% ammonium oxalate) for 5 min at 37 °C, centrifuged again and the resultant cell pellet was fixed in Carnoy's fixative (3 : 1 methanol, acetic acid). After centrifugation, the cells were resuspended in a small volume of fixative and dropped on to precleaned coded slides to avoid observer's bias.
The slides containing cells were stained with 0.125% acridine orange (BDH, England, Gurr Cat. No. 34001 9704640E) in Sorensen's buffer (pH 6.8), washed twice in the buffer. The slides mounted in Sorensen's buffer were observed under a fluorescent microscope equipped with 450–490 nm BP filter set with excitation at 453 nm (Carl Zeiss Photomicroscope III, Germany) using a 40 X neofluar objective.
A minimum of thousand binucleate cells with well preserved cytoplasm were scored from each culture and the frequency of micronucleated binucleate cells (MNBNC) was determined and the micronuclei identification was done according to the criteria of Countryman and Heddle [48]. The data regarding cell proliferation indices were also collected, where the frequencies of mono, bi and multi-nucleate (cell with more than two nuclei) cells were determined. All the experiments were repeated for confirmation of the results and the results of both experiments are combined and presented in the Tables and Figures.
The statistical analysis was carried out using one way ANOVA test. The data were fitted on apparent linear model ($\alpha + \beta X$ (scale of X axis) or linear quadratic ($Y = C + \alpha D + \beta D^2$) model.

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