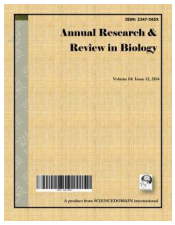

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Evaluation of the Clastogenicity of SJG-136, A Novel Pyrrolobenzodiazepine DNA Interstrand Cross-linking Agent, in Comparison with Nitrogen Mustard (HN2)

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Authors' contributions

This work was carried out in collaboration between all authors. Author FAD contributed in the design, realisation of the experimental part, wrote the protocol and interpreted the data as well as the writing and correspondence for the publication of the manuscript. Authors JAH and DET contributed in the design and funding the work. All authors read and approved the final manuscript

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ABSTRACT

Aims: SJG-136 is a new pyrrolobenzodiazepine used as an anticancer drug with high cytotoxicity against a panel of cancer cells and proved to produce interstrand crosslinks in the minor groove of DNA.
Methodology: In this work, SJG-136 (SG2000) was tested for its clastogenicity by calculating the rate of chromosomal aberrations (CAs), the mitotic index and the formation of micronuclei (MN) using the Chinese Hamster ovary (CHO) cell line.
Results: The results found showed that SJG-136 caused an increasing number of CAs especially chromatid and isochromatid breaks in comparison with nitrogen mustard (HN2) another well established anticancer drug extensively used as a DNA damaging agent, these CAs were shown to

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persist with time after treating cells with SGj-136. The mitotic index showed a delay in the cell cycle by more than 50% in cells treated with 0.1μM SJG-136 compared to a delay of 30-40% in cells treated with 10μM HN2. The MN test showed a clear increase of binucleated cells with MN with increasing concentrations of SJG-136 or HN2.

Conclusion: These findings suggest that SJG-136 appears to be stronger clastogenic agent compared with HN2 with high cytotoxicity and causing high number of CAs and MN.

Keywords: SJG-136; HN2; chromosomal aberrations; micronuclei; DNA-damage.

1. INTRODUCTION

Pyrrolo[2,1-c][1,4 benzodiazepines (PBDs) are a family of antitumour antibiotics that includes the natural products anthramycin and DC-81 [1-3]. They exert their cytotoxicity by covalently binding to the exocyclic CpG2. C2–NH2 group of guanine residues in the minor groove of DNA through their N10–C11 imine functionality [1-3]. This leads to a number of biological effects including the inhibition of transcription [4,5] and of enzymes binding to cognate sites [5,6]. The PBD monomers have significant *In vitro* cytotoxicity [7], and it has been demonstrated that joining two PBD moieties through a linker (via their C8-positions) leads to PBD dimers capable of interstrand DNA cross-linking [8-10]. One example of a PBD dimer, SJG-136 (NSC 694501, SG2000) [11], has undergone phase I clinical trials [12-15] and now undergoing phase II clinical trials [16]. It's a highly efficient interstrand cross-linking (ICL) agent that actively recognizes 5'-PuGATCPy-3' sequences in the DNA minor groove [12,17].

Nitrogen mustard (HN2) is a well-established anticancer drug which has been extensively studied as a DNA damaging agent [for review, 18]. HN2 is a bifunctional alkylating agent which forms a variety of adducts including monoadducts and cross-links. Monoadducts form principally at the N7 position of guanines and to a lesser extend at the N3 position of adenine. Cross-links can be intrastrand, interstrand (most favourably between guanine N7 positions in the sequence 5'-GNC-3'/3'-CNG-5' [19] and between DNA and proteins. The vast majority (~90%) of adducts are monoadducts, with inter- and intrastrand cross-links comprising only a small fraction of total lesions. Despite their rarity, there is good evidence that interstrand cross-links are the critical cytotoxic adducts produced by nitrogen mustards [20].

Chromosome aberration (CA) and micronucleus (MN) assays using Chinese hamster cell systems in culture have been widely used in primary screening for environmental mutagens and/or carcinogens. The CA assay as well as the MN assay belong to the standard three test battery for genotoxicity testing for pharmaceuticals recommended by the Fourth International Conference on Harmonization [21]. The MN arises from chromosomal fragments or whole chromosomes which are not incorporated into daughter nuclei during mitosis [22,23]. The MN assay using the cytokinesis block method is a fast and sensitive cytogenetic technique for evaluation of chromosomal damage in cells [24,25].

In this study, we assessed several cytogenetic parameters of genotoxicity of SJG-136 and HN2, namely induction of chromosome aberrations, mitotic index and micronuclei.

2. MATERIALS AND METHODS

2.1 Cell Culture

CHO cell line AA8 was obtained from Dr. M. Stefanini (instituto di Genetica Biochimica et Evoluzionistics, Pavia, Italy). Cells were maintained as a monolayer in F12-Ham medium (Sigma, Poole. UK) supplemented with 2mM glutamine and 10% foetal calf serum (Autogenbioclear, Wiltshire. UK). Cells were grown at 37°C in a 5% CO2 incubator and were harvested using trypsin-EDTA 1x solution (Autogenbioclear, Wiltshire. UK).

The CHO cell line has a stable aneuploid karyotype with modal number of chromosomes of 21.

2.2 Drugs

SGJ-136 was synthesised as described [11], the stock solution was prepared in DMSO (Sigma, Poole. UK) and stored at -20°C.

Analytical-grade mechloroethamine (HN2) (Sigma, Poole. UK) was dissolved in culture medium F12-Ham directly before treatment.

The structures of the drugs are shown in Fig. 1.

2.3 Cytotoxicity Assay

Cytotoxicity was determined following a 1 hour drug incubation using the Sulforhodamine B (SRB) growth inhibition assay, described in details previously [26,27]. Growth inhibition was measured by quantifying the number of cells three days after drug exposure.

2.4 Micronucleus Assay

The MN study was performed accorded by GLP guidelines.

2.4.1 Treatment Procedure

Cells were cultured in 90 mm plastic Petri dishes 24 hours prior to drug treatment; SGJ-136 or HN2 were added to the culture at different concentrations: 0.01, 0.05, 0.1, 0.5 µM for SGJ-136 and 1, 3, 10, 20 µM for HN2. After 1 hour treatment in serum free medium, cells were washed and cytochalasin B (Sigma, Poole. UK) was added at a final concentration of 4.5 µg/ml in full medium. Cells were incubated for further 28 hours at 37°C. Cells were trypsinized and centrifuged at room temperature, a hypotonic solution (water: medium (4:1 v/v)) was added to the pellet for 10 minutes at room temperature. Cells were fixed twice in ice cold methanol for 10 minutes, spread onto dry clean slides and air-dried.

2.4.2 Slide staining

Slides were stained for 20 minutes with 0.5% acridine orange (Sigma, Poole. UK) then washed with distilled water for another 20 minutes. The slides were air-dried. Each experiment was performed in duplicate.

2.4.3 Scoring

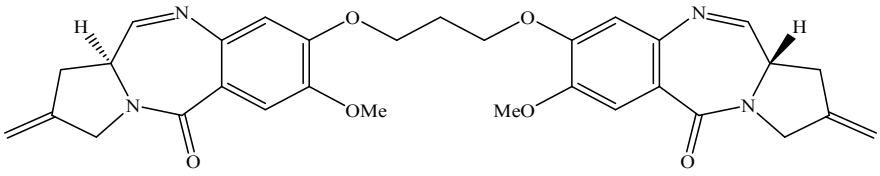
Each experiment was performed in duplicate, for each drug tested at the different concentrations studied, 1000 cells were examined for measuring the micronucleus formation using a fluorescence microscope. Only cells with a binucleated nucleus and a well preserved nuclear membrane were scored.

2.5 Chromosome Aberrations

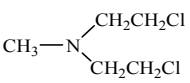
The CA study was performed accorded by GLP guidelines.

2.5.1 Treatment procedure

CHO ($5x10^5$) cells were seeded in 90 mm plastic Petri dishes 23 hours before treatment, then different concentrations of the test chemicals were added to each Petri dish. SJG-136 was used at 0, 0.01, 0.05, 0.1, 0.2 µM and HN2 was used at 0, 1, 3, 10, 20 µM for 1 hour in serum free medium. Cells were left in fresh full medium for 24 and 43 hours at 37°C before harvesting.



SJG-136



Mechlorethamine

Fig. 1. Structures of the PBD dimer SJG-136 and the nitrogen mustard mechlorethamine

During the last 2 hours of culture, colcemid (Sigma, Poole. UK) was added to the medium at a final concentration of 0.1 µg/ ml. At the end of this time, cells were collected by trypsin/EDTA treatment, treated with hypotonic solution (75 mM KCl) for 10 minutes at 37°C and fixed twice in methanol: Acetic acid (3:1 v/v) at room temperature. Cells were then dropped onto ice-cold glass slides, air-dried and stained with 3% Giemsa solution in bidistilled water for 30 minutes then washed and left to dry overnight. Each experiment was performed in duplicate.

2.5.2 Scoring

Scoring of chromosome aberrations (CAs) was performed in 100 well-spread metaphases per test point. Only metaphases containing 20-22 chromosomes were scored. A chromatid gap (CG) was defined as an achromatic or unstained constricted region on one chromatid, the size of which is equal or smaller than the width of the chromatid, while a chromosome gap or isochromatid gap (ICG) was scored as a gap present on both chromatids, either in the same position (isogap or isolocus) or at different positions along the chromosome length. A chromatid break (CB) is an achromatic region in one chromatid larger than the width of the chromatid. It may be either aligned or unaligned with the chromatid. The displacement of the broken chromatid fragment results in a terminal deletion while a chromosome break or isochromatid break (ICB) is observed as breaks in both chromatids. A fragment was defined as a single chromatid without an evident centromere [28,29]. Other aberrations were reported as translocation, deletions, rings, dicentric and acentric chromosomes.

The mitotic index (MI) was defined as the number of metaphases counted in 2000 cells scored on the slide.

2.6 Statistical Analysis

The One-Way ANOVA test was used for the data analysis of the CAs while the t-test was used to evaluate the micronuclei formation induced by the test drugs.

3. RESULTS

3.1 Growth Inhibition Assay

The data presented in Fig. 2 show the sensitivity of AA8 cells to SJG-136 and HN2 using SRB growth inhibition assay. The IC_{50s} (concentration of drug to inhibit growth by 50%) showed a high level of cytotoxicity caused by SJG-136 (0.28 µM) (Fig. 2A) compared to HN2 (18.56 µM) (Fig. 2B), these results show that SJG-136 is 66 fold more potent than HN2.

3.2 Micronucleus Formation

The cytokinesis-block micronucleus assay is an established cytogenetic method for the measurement of chromosome breakage and loss in nucleated cells [25]. By blocking cytokinesis using cytochalasin-B it is possible to specifically score micronuclei (MN) in once divided cells, which are recognized by their appearance as binucleated cells (Fig. 3).

CHO-AA8 cells were treated with SJG-136 or HN2 for 1 hour and cells were left for another 28 hours at 37°C in the presence of cytochalasin B. The numbers and the means ± standard deviation (SD) of binucleated cells with no MN and binucleated cells with 1, 2, or more than 2 MN were scored at each drug concentration and in the control (tables 1and 2). For both drugs, the results show that the number of binucleated cells without MN decreased significantly ($P < 0.05$) with increasing concentrations of the test chemicals. For SJG-136, a significant difference in the number of MN formed was detected after treating the cells with 0.01 µM in comparison with the control (2 fold higher after treatment with SJG-136 at 0.01 µM) and at 0.5 µM it was 5 times higher than the MN scored in the untreated cells (Table 1).

For HN2, the same pattern of MN distribution was shown with a 2 fold increase in MN formation after treatment with 1 µM HN2 and 4.7 times more MN scored in binucleated cells treated with 20 µM HN2 than in the control (Table 2).

Fig. 4 illustrates the frequencies of micronuclei formation induced by both drugs showing a high frequency of MN formation in both SJG-136 (Fig. 4A) and HN2 (Fig. 4B).

3.3 Chromosome Aberrations

CHO-AA8 Cells were treated for 1 hour with SJG-136 or HN2 and then left at 37°C for one or two rounds of DNA replication before harvesting. A total number of 100 metaphases were scored and represented as the percentage of CAs per metaphase classified in seven classes, CB, ICB,

CG, ICG, rings, dicentric and acentric chromosomes and other types of aberrations (OTA) including translocations, deletions and fragments of chromosomes. Because of the unknown nature of CG and ICG, we did not include them in the percentage of total aberrations (TA).

SJG-136 induced a significant increase in CAs formation; both when gaps were included and excluded (Table 3). After only 0.01 µM drug induction, a 2.6 fold increase of the frequency of CAs was shown in comparison with the control; after 0.05 µM drug treatment, CAs increased by 5.3 fold and at 0.1 µM the frequency of CAs was very high about 10 fold the aberrations scored in the untreated cells, at 0.2 µM SJG-136 it was impossible to score metaphases because of the high number of pulverisations. These effects of SJG-136 were dose-dependent ($P < 0.05$). After 2 rounds of DNA replication, CHO-AA8 cells showed more increasing numbers of CAs with increasing concentrations of SJG-136. At 0.01 µM, the frequency of aberrations reached more than 3.5 times the values found in the control. The figures increased to more than 10 fold at 0.05 µM and 14 fold at 0.1 µM (Table 3).

Fig. 5 shows images of a pulverized chromosome after the treatment of CHO cells with 0.2 µM SJG-136 compared with an untreated CHO cell.

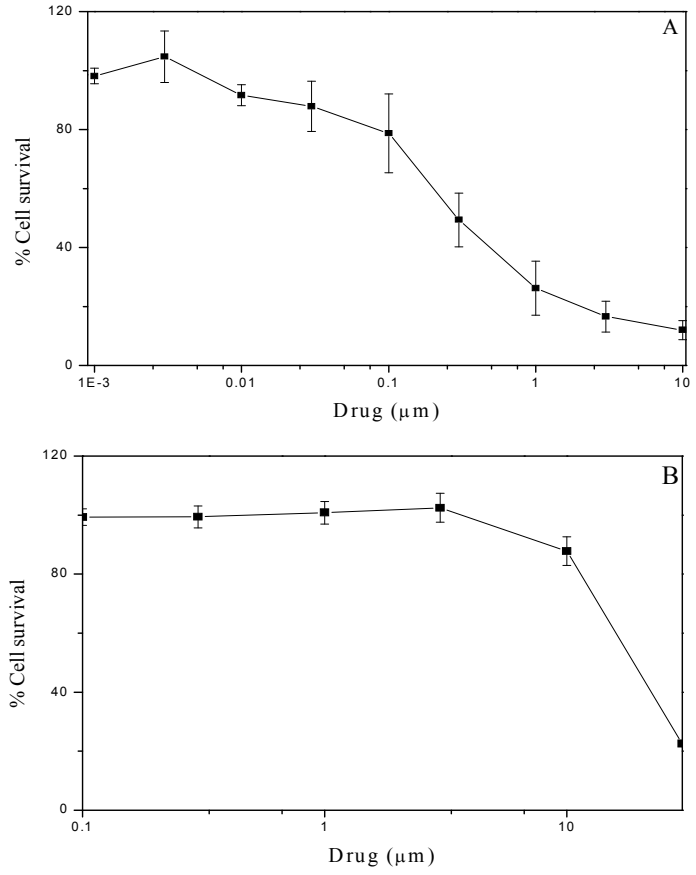


Fig. 2. Survival of CHO AA8 cells following 1 h of exposure to increasing concentrations of SJG-136 (A) and HN2 (B). All results are mean of 3 independent experiments, and error bars show the standard error of the mean

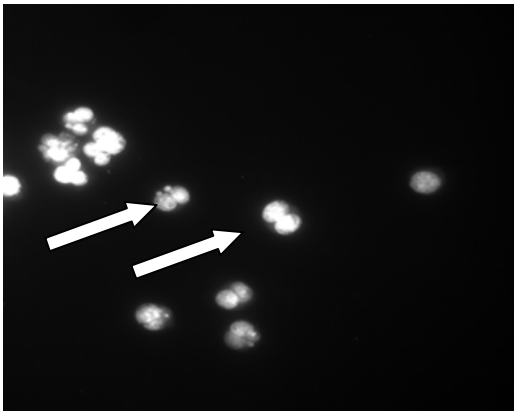


Fig. 3. Image of binucleated cells with micronuclei treated with 0.05 µM SJG-136 for 1hour before proceeding with the micronucleus assay. Arrows show binucleated cells with micronuclei (Magnification 40x)

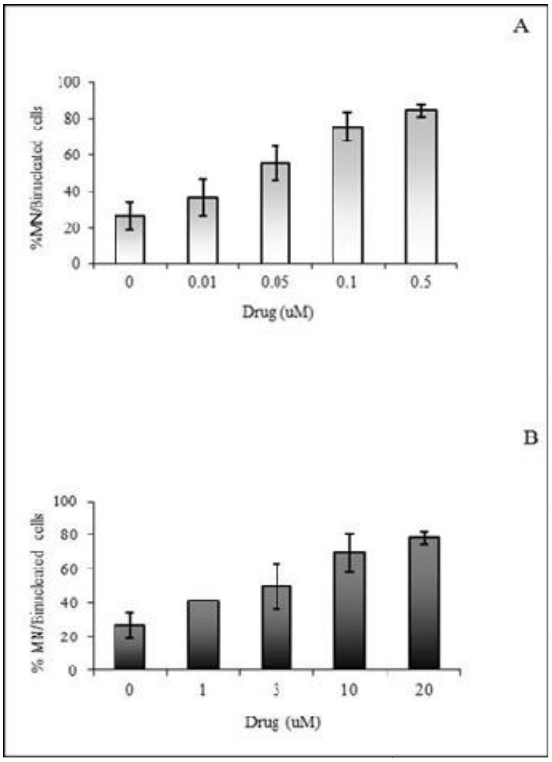


Fig. 4. Frequency of micronuclei induced in CHO-AA8 cells after a 1 hour treatment with increasing concentrations of SJG-136, A; and HN2, B. In each experiment, 1000 binucleated cells were counted. Data shown are the average of two independent experiments and error bars show the standard error of the mean

After 24 hours post-treatment, CAs formed after 1 µM HN2 treatment did not show any significant difference with the control cells. At 3 µM, the percentage of TA per cell doubled, at 10 µM it increased to 6 fold and at 20 µM it showed an 8 fold increase in comparison with the control (Table 4).

After 2 round of DNA synthesis, the cells seemed to be repairing the damage caused by the drug showing a clear decrease in the formation of CAs from 6 times after one round of DNA replication to 2 fold after 2 rounds of DNA replication at 10 µM dose and from 8 fold to 2.5 fold at 20 µM dose (Table 4).

4. DISCUSSION

Experimental analyses have shown that DNA double strand breaks (DSBs) are the principal lesions in the process of chromosome aberrations (CAs) formation [30-32]. The majority of chemical mutagens are not able to induce DSBs directly but lead to other lesions in chromosomal DNA which, during repair or DNA synthesis may give rise to DSBs and eventually to CAs [33]. DSBs are induced in response to interstrand cross-links [26].

SJG-136 is an interstand cross-linking (ICL) agent that forms cross-links rapidly in cultured cells as well as in xenografts [12]. The repair of DNA interstrand cross-links is poorly understood in mammalian cells but it appears to require components of both nucleotide excision repair (in particular XPF and ERCC1) and homologous recombination [26,34]. In a panel of normal and DNA repair defective Chinese hamster ovary cell lines, SJG-136 was highly cytotoxic compared with melphalan another nitrogen mustard drug [35]; this is in agreement with our results showing that SJG-136 was highly cytotoxic compared to HN2 with an IC_{50} dose 66 times lower than HN2. The CAs assay showed that the number of cells with different types of aberrations increased significantly in cultures treated with increasing concentrations of SJG-136 or HN2. Chromatid breaks and isochromatid breaks were the major aberrations induced by both drugs. SGJ-136 seems to cause more damaging effects on the cells at very low doses in comparison with HN2 (6.6 at 0.1 µM SJG-136 versus 5.3 at 20 µM HN2) taking in consideration that the IC_{50} concentration of SGJ-136 is 0.28 µM compared to 18.5 µM for HN2. After 2 rounds of DNA synthesis, CHO-AA8 cells were not able to repair the damage caused by SGJ-136 showing increasing figures of chromosome aberrations;

these results could be explained by the fact that ICL lesions caused by SGJ-136 form rapidly and persist in comparison with those produced by more conventional DNA cross-linking agents such as the nitrogen mustards, also, these lesions were shown to be more difficult to remove from human tumor cells [12]. On the other hand, with HN2, there was a clear decrease in chromosome aberrations formation as a consequence of a rapid removal of the lesions to completely disappear after 43 hours at low concentrations (1 and 3 µM HN2). It was shown in a previous report by De Silva et al. that the ICL-associated DSBs formed by the nitrogen mustard HN2, using the same conditions as in this study were repaired rapidly with complete recovery after 24 hours [26]. Our results showed that most of the CAs induced by medium to higher concentrations of HN2 where removed after 43 hours.

The percentage of the mitotic index relative to untreated cells showed a delay in the cell cycle by more than 50% in comparison with the control for SJG-136 at 0.1 µM compared to 30-40% for HN2 at 10 µM giving a clear indication of the clastogenicity of both drugs. The effect of SGJ-136 on the cell cycle was examined in K562 cells after a 1 hour drug-exposure showing an accumulation of cells in the G2-M phase after 24 h [12]. Previous studies showed that SJG-136 lesions cause minor distortion of the DNA helix (11), this could be a reason for the non recognition of the lesions by the repair mechanism complexes such as XPF-ERCC1 and the homologous recombination factors XRCC2 and XRCC3 involved in maintaining chromosome stability during cell division [35] than is HN2 [34-36].

The micronucleus test has been adopted for use in human cells to evaluate the clastogenic effects of drugs before they are commercialised [37]. Our results show that the number of binucleated cells without MN was significantly decreased in both SJG-136 and HN2 treated cells compared with the untreated cells, at the same time, the number of binucleated cells containing MN, as well as, the frequencies of MN was increased significantly compared with the control. As the MN are small chromatin-containing bodies arising from chromosome fragmentation by breaks or deletion, the results of MN formation confirm the results found of CAs indicating the clastogenicity effect of both SJG-136 and HN2. At equitoxic doses, similar effects on the MN formation were shown with both drugs. At the chromosome level, CHO AA8 cells showed more figures of aberrations with SJG-136 than with HN2.

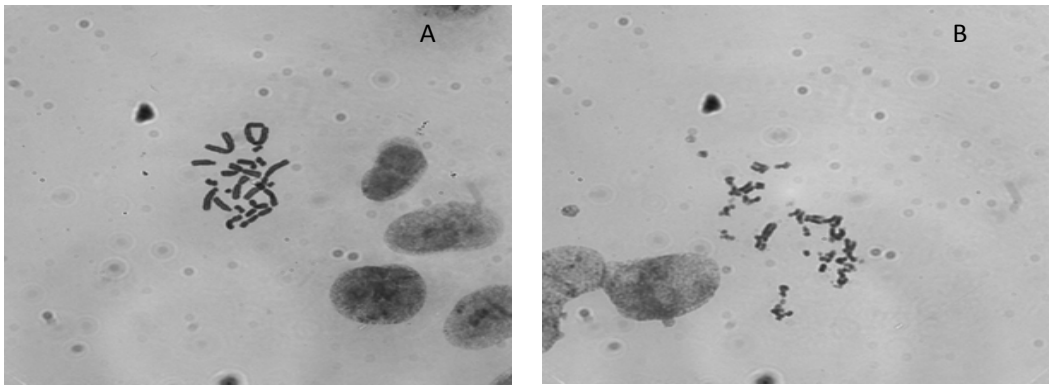


Fig. 5. Chromosomal aberration test. CHO ($5x10^5$) cells were cultured in 90 mm plastic Petri dishes 23 hours before treatment, cells were treated with 0.2 µM SJG-136 for 1 hour in serum free medium, then left in fresh full medium for another 24 hours at 37°C before harvesting. Colcemid was added to the medium at a final concentration of 0.1µg/ml during the last 2 hours of culture. Cells were collected by trypsin/EDTA treatment, treated with a 75 mM KCl solution for 10 minutes at 37°C and fixed twice in methanol: acetic acid (3:1 v/v) at room temperature. Cells were then dropped onto ice-cold glass slides, air-dried and stained with 3% Giemsa solution in bidistilled water for 30 minutes then washed and left to dry overnight. A. control CHO cells; B. 0.2 µM SJG-136 treated cells showing a pulverized chromosome (Magnification 40x)

Table 1. Effect of SJG-136 on the number of binucleated cells containing micronuclei (MN)

Drug concentrations	No. Cells	Binucleated cells (no MN)		Binucleated cells with 1 MN		Binucleated cells with 2 MN		Binucleated cells with More than 2 MN		Total number of Binucleated cells with MN	
		No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD
Control	2000	1668	834±32.53	280	140±24.04	38	19±4.95	14	7±3.54	332	166±32.53
0.01uM SJG-136	2000	1270	635±48.79*	576	288±31.11*	116	58±19.80*	38	19±2.12*	730	365±48.79*
0.05uM SJG-136	2000	892	446±46.67*	704	352±4.24*	272	136±28.28*	132	66±22.63*	1108	554±46.67*
0.1uM SJG-136	2000	496	248±25.46*	600	300±2.83*	388	194±7.07*	516	258±21.21*	1504	752±39.6*
0.5uM SJG-136	2000	310	155±17.68*	528	264±4.24*	516	258±16.97*	646	323±4.95*	1690	845±17.68*

**P < 0.05, significantly different from the control*

Table 2. Effect of mechlorethamine on the number of binucleated cells containing micronuclei (MN)

Drug concentrations	No. cells	Binucleated cells (no MN)		Binucleated cells with 1 MN		Binucleated cells with 2 MN		Binucleated cells with more than 2 MN		Total number of binucleated cells with MN	
		No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD	No.	Mean±SD
Control	2000	1668	834±32.53	280	140±24.04	38	19±4.95	14	7±3.54	332	166±32.53
1uM SJG-HN2	2000	1168	584±0.00*	704	352±41.01*	102	51±33.23*	26	13±7.78*	832	416±0.00*
3uM SJG- HN2	2000	1010	505±67.18*	694	347±34.65*	200	100±22.63*	96	48±9.9*	990	495±67.18*
10uM SJG- HN2	2000	612	306±56.57*	644	322±15.56*	330	165±17.68*	414	207±54.45*	1388	694±56.57*
20uM SJG- HN2	2000	438	219±19.9*	430	215±0.71*	596	298±19.8*	536	268±1.41*	1562	781±19.09*

**P < 0.05, significantly different from the control*

Table 3. Frequencies of chromosome aberrations induced in CHO-AA8 cells treated with SJG-136

Drugs	No. Cells	Aberrations / 100 cells								%TA-G/Cell (Mean±SD)	Relative MI (%)
		CB	ICB	CG	ICG	Ring	Dic	Acent	OTA		
24 h post-treatment											
Control	200	1.5	1	0.5	0	0	1.5	1	1.5	0.65±0.35	100
0.01uM SJG-136	200	4.5	5	1	0	0.5	2.5	2	2.5	1.7±0.14*	59.86
0.05uM SJG-136	200	6	9.5	4	0	1	3.5	6	8	3.4±0.0*	55.22
0.1uM SJG-136	200	15.5	20	7	0	0.5	4	5.5	20	6.6±0.35*	46.05
43 h post-treatment											
Control	200	2.5	1	1	0.5	0.5	2	1.5	2.5	0.9±0.31	100
0.01uM SJG-136	200	10	5.5	3	0	2.5	2.5	4	9.5	3.3±0.23*	57.23
0.05uM SJG-136	200	21.5	25	7	0	4	6	5	28.5	9.2±1.6*	43.23
0.1uM SJG-136	100	38	40	26	0	4	5	6	34	12.6±0.39*	15.75

CA, chromatid aberrations; ICA, isochromatid aberrations; CG, chromatid gap; ICG, isochromatid gap; Dic, Dicentric chromosomes; Acent, Acentric chromosomes; OTA, other types of aberrations; TA-G, total aberrations excluding gaps; MI, mitotic index

** P < 0.05, significantly different from the control*

Table 4. Frequencies of chromosome aberrations induced in CHO-AA8 cells treated with mechlorethamine

Drug concentrations	No. of cells	Aberrations / 100 cells								%TA-G/Cell (Mean±SD)	Relative MI (%)
		CB	ICB	CG	ICG	Ring	Dicent	Acent	OTA		
24 h post-treatment											
Control	200	1.5	1	0.5	0	0	1.5	1	1.5	0.65±0.35	100
1uM mechlorethamine	200	3.5	1.5	1	0	0	2	1	2	1.0±0.03	75.34
3uM mechlorethamine	200	4	2.5	1.5	0	0	1.5	3	3	1.4±0.0*	63.11
10uM mechlorethamine	200	7.5	12	4.5	0.5	0.5	3	6	11	4.0±2.83*	61.04
20uM mechlorethamine	200	11	16	2.5	0	0	4	7	14.5	5.3±4.6*	36.68
43 h post-treatment											
Control	200	2.5	1	1	0.5	0.5	2	1.5	2.5	0.9±0.31	100
1uM mechlorethamine	200	1.5	1.5	1	0	0.5	1.5	1	2.5	0.8±0.1	88.6
3uM mechlorethamine	200	2	2	1.5	0	1	1	0.5	3.5	1.0±0.37	69.02
10uM mechlorethamine	200	4	5	2	0	1	1.5	3	4	1.8±0.09*	50.72
20uM mechlorethamine	100	5	3	1	0	4	3	2	7	2.3±0.1*	27.6

CA, chromatid aberrations; ICA, isochromatid aberrations; CG, chromatid gap; ICG, isochromatid gap; Dic, Dicentric chromosomes; Acent, Acentric chromosomes; OTA, other types of aberrations; TA-G, total aberrations excluding gaps; MI, mitotic index.

** P < 0.05, significantly different from the control*

5. CONCLUSION

We can conclude, on the basis of our results, that SJG-136 appears to be stronger clastogenic agent than HN2 by being more cytotoxic and by causing high number of CAs and MN.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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