[BIO42] Application of comparative genomic hybridization and fluorescence *in situ* hybridization on human glioma cell lines treated with bis[*S*-methyl-*β*-*N*-(2-furylmethylketone) dithiocarbazato] cadmium(II)

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Introduction

Gliomas are the most common primary tumors which arise from cells of the brain itself rather than metastazing to the brain from another location in the body. It can be slow growing (low grade, grades 1 and 2) or rapidly growing (high grade, grades 3 and 4). For instance, diffuse and fibrillary astrocytomas are divided histopathologically into three grades of malignancy: World Health Organization (WHO) grade II astrocytomas, WHO grade III anaplastic astrocytoma and WHO grade IV glioblastoma multiforme (GBM) (Otto, 2001).

It seem to have the genomic alterations at the chromosome level somewhere in DNA that are characteristic of each type of tumor and most stages of progression but not much is known. Because of this factor, it present a major therapeutic challenge, there is a strong rationale for examining the efficacy of novel chemotherapeutic strategies as a means for enhancing disease control (Devaux et al., 1993).

An understanding of these specific genetic alterations will give information to the oncologists in doing cancer therapy, which is currently based only on cancer classification such as for gliomas, one of the major groups in brain tumor.

Thus, the aim of this study were to determine the EC_{50} value for bis[*S*-methyl-*β*-*N*-(2-furylmethylketone)dithiocarbazato] cadmium (II) (SMDB-Cd) in order to screen the cytotoxic effects on human gliomas cell lines then to examine and compare the deletion and amplification patterns in whole chromosomes in normal DNA (reference) and DNA from gliomas cell lines (test) before and after treatment with SMDB-Cd using CGH and to confirm that p53 gene is one of the genes involved in progression of the gliomas by using p53 FISH probe.

Materials and methods

Cell lines and culture medium

Three different stages of human glioma cell lines were used; A172 (glioblastoma), U87 MG (astrocytoma grade III) and T98G (glioblastoma multiforme) were all purchased from American Type Culture Collection (ATCC), USA. A172 was cultured in DMEM and U87MG and T98G in EMEM. All media was supplemented with 10% Fetal Bovine Serum (FBS), 100U/ml and 100mg/ml Penicillin-Streptomycin solution and 2mM L-Glutamine (4mM L-glutamine).

Compound preparation

The SMDB-Cd compound was dissolved in 0.05% dimethyl sulfoxide (DMSO, pH 4.7) (Amresco, USA) and diluted with DMEM 5% FBS and EMEM 5% FBS for treatment on U87MG, A172 and T98G. Tamoxifen (SIGMA, USA) a commercially available drug which is used very common to treat brain cancer was used as the control and was prepared by using the same method.

In vitro cytotoxicity assay

Investigation on the effects of SMDB-Cd on cell growth inhibition employed the use of MTT assay. Cells (1 X 10^5 cells/well for HCN-2, A172, U87MG and T98G cells) were plated with nutritional medium (DMEM 5% FBS or EMEM 5% FBS) in 96 well plates. The total volume for each well was 100µl. After 24 hours, cells were treated with different concentrations of SMDB-Cd and tamoxifen (control) for 72 hours. The plates contain 100 microliters (µl) of media in each 96 well with addition of the compound and media to a total volume of 200 microliters. After treatment for 72 hours, twenty microliters of MTT (Sigma, USA) solution [5 mg/ml in 1X PBS] was added to each well and then wrapped with aluminium foil. The plates

were then reincubated at 37°C in 5% CO_2. After 4 hours of incubation, the medium was discarded and 200 µl of lysis buffer, DMSO (pH 4.7) (Amresco, USA), was added to each well, followed by 15 minutes incubation at 37°C in 5% CO_2 to dissolve the reduced MTT crystal. The absorbance of the samples was then measured by an ELISA reader (DynexMRX II) at 530nm. Each of the experiment was repeated at least 3 times. The EC_{50} values were then analyzed using T-test to test for the significance and data were given as mean ± standard error means (SEM).

Comparative genomic hybridization (CGH)

High-quality metaphases chromosome spreads from a karyotypically normal male for CGH were commercially bought (Vysis). Briefly, DNA from untreated and treated cell lines with SMDB-Cd and peripheral blood cells was extracted using a *Dneasy™ Tissue kit* (Qiagen) and *QIAamp Blood Mini kit* (Qiagen). Tumor and reference DNA were labeled with SpectrumGreen-dUTP and SpectrumRed-dUTP (Vysis) respectively, using a nick translation kit (Vysis). Both DNA were simultaneously hybridized for 16-24 hours at 37°C in HYBrite™ system (Interscience, Germany). Chromosome counterstaining was achieved by using DAPI/antifade (Vysis).

Fluorescence in situ hybridization (FISH)

Fish study was performed on those cell lines in order to confirm the CGH findings. Metaphases chromosome spreads were prepared from untreated and treated cell lines and peripheral blood cells according to standard protocols. FISH was carried out using a unique sequence probe for the p53 gene (Vysis). The experiments were performed according to the protocols supplied by probe's manufacturer.

Digital image analysis

CGH and FISH results were analyzed using Leica fluorescence microscope (Leica System, Germany) equipped with appropriate filters. Between 10 and 15 mwtaphases were chosen for image analysis based on their high fluorescence intensity and uniform hybridization. Gray-level images were captured separately for each fluorochrome (DAPI, SpectrumGreen and SpectrumRed). Karyotyping was performed based on DAPI banding and was achieved by inverting the DAPI image. For CGH, green-to- red fluorescence intensity ratio profiles were calculated after background correction and normalization of the green-to-red ratio for each metaphase to 1.0. Mean ratio profiles for each chromosome were determined after data from all analyzed metaphases were combined. Ratio values that were under 0.8 and over 1.2 corresponded respectively to loss and gain of DNA sequences in the DNA study. In FISH analysis, only yellow and blue images were collected.

Results

Cytotoxic activity of the SMDB-Cd on human glioma cell lines

The cytotoxic activity of the SMDB-Cd was found the most effective on the U87MG cell line, followed by T98G, then A172 and lastly HCN-2. These MTT results demonstrate that the U87MG cell line was more sensitive to the SMDB-Cd compared to the others cell lines. EC_{50} of tamoxifen on A172, U87MG and HCN-2 were 7µl/ml and 5µl/ml 6µl/ml respectively (Table 1) which is comparable to that reported in the literature for U87MG was at 5.57 µg/ml (Brondani *et al*, 1999). Figure 1 shows the cell viability for each cell line treated with Tamoxifen concentrations for the control. Meanwhile Figure 2 shows the cell viability for each cell line treated with SMDB-Cd. Graph line for each figure representing 72 hour treatment. Therefore, SMDB-cd is more potent than tamoxifen.

TABLE 1 EC_{50} of the synthetic compound, SMDB-Cd compared to tamoxifen on HCN-2, A172, U87MG and T98G cell lines.

Cell lines	Tamoxifen	SMDB-Cd
HCN-2	6 µg/ml ±0.10	1.5 µg/ml 0.145
A172	7 µg/ml ±0.15	0.7 µg/ml ±0.02
U87MG	5 µg/ml ±0.09	0.3 µg/ml±0.006
T98G	4 µg/ml ±0.44	0.4 µg/ml ±0.028

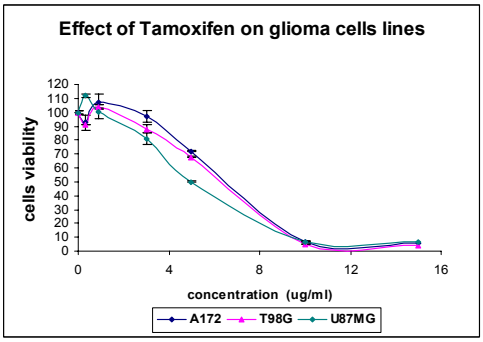

FIGURE 1 Cytotoxicity effect of tamoxifen on glioma cell lines A172, T98G and U87MG.

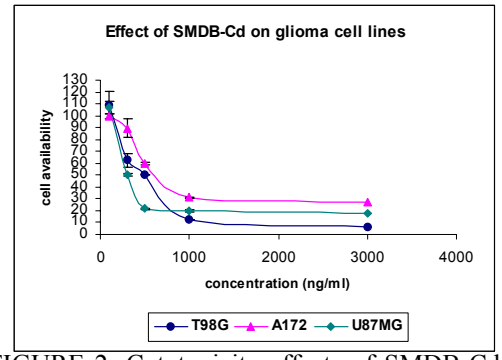

FIGURE 2: Cytotoxicity effects of SMDB-Cd on glioma cell lines

CGH

In untreated cell lines, the most frequently observed alteration involved gains of 7p and 12q which were detected in all the cell lines. Losses of 9p, 10, 17p and 19q were only observed in A172 and U87MG. In T98G, the losses region only involved chromosome 9p and 10 (Figure 3). After treatment with the SMDB-Cd, there are two group of observation is done in the cell lines. Attention is given to the regions that show still remains which means not affected by the compound or no changes observed in each cell line and the regions that may affected by the compound (Figure 4).

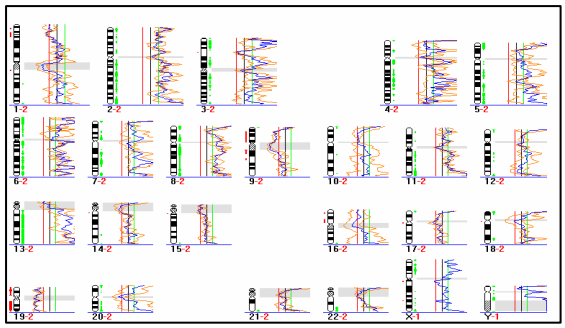

FIGURE 3 The average green to red fluorescence ratio profiles of each cell line before treatment with the SMDB-Cd computed from the 10 ratio images.

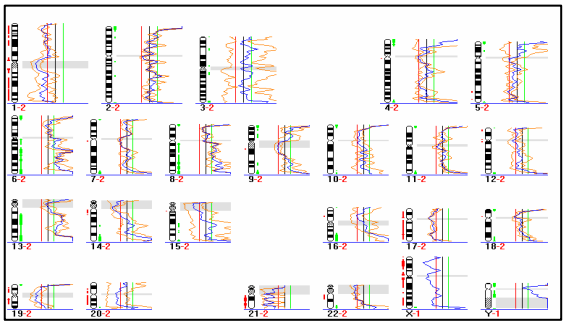

FIGURE 4 Summary of genetic imbalances detected by CGH in each cell line after treatment with SMDB-Cd.

FISH

FISH analysis found significance deletion of p53 in untreated A172 and U87MG cell lines. Its means there were abnormalities in p53 involved in those gliomas cell line that agreed with the CGH results. In T98G, two copy of p53 gene was the most frequent observed. However, examination of the p53 level in treated cell lines showed the increasing of p53 signal appeared in T98G. Two copies of p53 signals in a normal cell are presented in figure 5a. Figures 5b, 5c, 5d and 5e show the examples of p53 signal in untreated and treated cell lines.

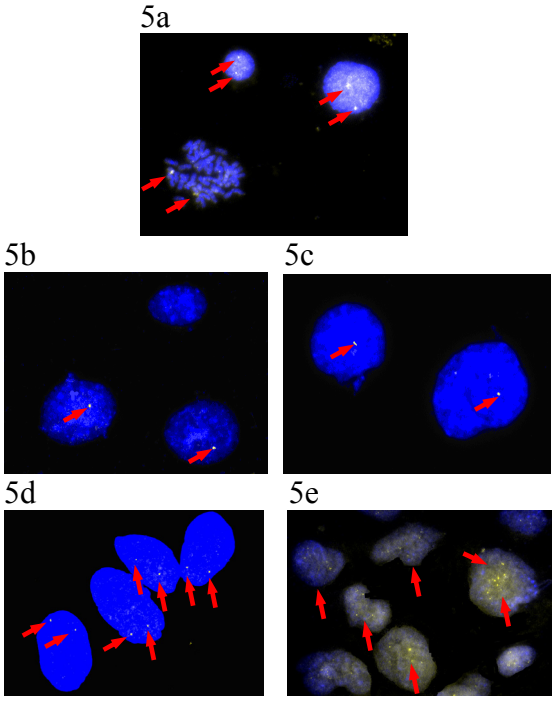

FIGURE 5 Results of FISH experiments using Vysis LSI p53 (17p13.1) probe (yellow) performed on chromosome preparations of samples (red arrows). 5a) Normal peripheral blood lymphocytes cells with two copies of the p53 gene. 5b) Untreated A172 and U87MG with one copy of the p53 gene. 5c) Treated A172 and U87MG with one copy of the p53 gene. 5d) Untreated T98G with two copies of the p53 gene. 5e) Multiples signal of p53 gene in each T98G cell after treatment with SMDB-Cd (red arrow) (100X magnification).

Discussion

Results of the present study revealed that the SMDB-Cd appeared to be more active and also showed more potent activity (in terms of EC_{50}) as compared to tamoxifen, indicating that this compound could be a possible candidate against glioma. A compound is indicative of weak cytotoxic activities when the EC_{50} values are more than 10 – 25 µg/ml (Shier, 1991). A compound is chemotherapeutically significant and actively cytotoxic when the EC_{50} < 4 µg/ml (Geran *et al*., 1972 and Shier, 1991). The EC_{50} value of the SMDB-cd is in nanogram concentrations, which is well below 4 µg indicating the compound is chemotherapeutically significantly (Geran et al; 1972; Shier, 1991).

The CGH with spectrum-green labeled tumors cell lines DNA and normal spectrum-red labeled reference DNA revealed frequent gains of DNA at 7p and 12q, and losses of chromosome 9p, 10, 17p and 19q indicating that these regions contain candidate tumor suppressor genes involved in gliomas. These results are concordant with previously reported genomic aberrations in gliomas (Liau *et al*., 2001). Accordingly, all these regions may be significant in the development or progression of gliomas, and the cell lines examined are likely to be useful resources for genetic analysis. Recent work of focusing on the gains and losses of these regions has been shown to play a critical role in the oncogenesis of malignant tumors (Liau *et al*., 2001).

The expected high green to red ratio at the regions above that corresponds to the some genes such as: *EGFR, MDM2, p53* and *PTEN,* loci are clear in all cell lines. The height of the peak does not quantitatively reflect the level of amplification because the fluorescent signal spreads over a region of the chromosome that is larger than the length of the amplicon (Kallionemi *et al*., 1992). This is apparently a result of the target DNA in the denatured chromosomes. The CGH technique provides a means to detect and map such sequences. Several amplified loci were mapped and many at regions of the genome where amplification had not been suspected. Thus, a large variety of genes may be amplified during cancer initiation and progression (Kallioniemi *et al*., 1992).

The region of 7p12-14 was the most common region showing DNA copy number gain in all cell lines. Liau *et al*. (2001) stated that an increased copy number at 7p12-14 is the aberration occurring most frequently in gliomas. This region contains the EGFR gene for a 170 kDa protein. EGFR is a transmembrane tyrosine kinase receptor that binds to at least two ligands: EGF and TGF-α. Careful study of EGFR overexpression/amplification and p53 mutations has demonstrated that primary glioblastomas are associated with EGFR overexpression/amplification, but that p53 mutations are rare in this subgroup. This is in agreement with this study where a gain of 7p12-14 is clearly shown in all 10 metaphase for each cell line but the deletion of p53 was only shown in U87MG and A172 (by CGH). These results suggest that T98G may not arise from the lower grade astrocytomas and U87MG and A172 may arise through progression from lower grade astrocytomas.

In this study, aberrations of the p53 gene on chromosome 17 were observed in all three cell lines. In A172 and U87MG, none or only one copy of the p53 gene was detected before treatment compared to the normal control that have two copies of the p53. This finding implies that both of these cell lines were found to have deletion on one or both copies of p53 gene. However, in T98G, some interphase cells show both copies of the p53 signal that means this cell line has a wild type copy of p53. After treatment with SMDB-Cd, the p53 signal was shown to have more than two signals in T98G. This amplification of p53 which appears in the treated T98G may be due to the action of the cadmium complex, SMDB-Cd on the inhibition of cell growth and lead to DNA damage. SMDB-Cd may have inhibited division of cells that lead to the accumulation of p53 gene in a cell (figure 5e).

This finding is in agreement with a previous report which showed that non-repaired drug-induced DNA damage may result in: i) inhibition of DNA synthesis, ii) alteration in gene expression, iii) induction of apoptosis and iv) loss of genetic information. Generally, it has been assumed that DNA lesions block DNA synthesis and prevent cellular replication. Even if the lesion can be by-passed, persistence of the lesion in one cell and possible loss of genetic information in both daughter cells may eventually be lethal. DNA lesions may also have pronounced effects on gene expression, affecting RNA elongation or even the binding of polymerases and transcription factors (Yarnold *et al*., 1993).

Differential tolerance of DNA damage could account for differences in sensitivity to a chemotherapeutic drug, either between normal and neoplastic cells, or between sensitive and resistant tumors. Many features of cell death induced by anticancer drugs, such as chromatin condensation and activation of DNA endonucleases, are similar to apoptosis, often called programmed cell death (Eastman *et al*., 1990).

Recent evidence has suggested a role for the p53 gene in response to DNA damage (Lane, 1992). Genetic alteration of p53 is one of the most frequently observed changes in human neoplasia and the p53 gene has been implicated both as a proto-oncogene and as a tumor suppressor gene. The level of p53 gene has been shown to be increased in cells after treatment with a variety of DNA damaging agents, including chemotherapeutic agents. In the case of ionizing radiation, p53 levels alter in temporal association with a G_1 cell cycle arrest in cells with wild type p53; cells with mutant p53 do not arrest at G_1 (Kastan *et al*., 1991). It has been suggested that wild type p53 has a role in the inhibition of DNA synthesis following DNA damage, and alteration of p53 may cause a failure of this inhibition, an increased sensitivity to DNA damage and an increased mutation rate in the genome (Yarnold *et al*., 1993)

It has been postulated that the drug sensitivity of certain tumor types may be due to deficiencies of DNA repair in these cells that acquired during development of the tumor. If defective DNA repair processes are involved in tumor development, identification of the particular defect in tumors and hence the DNA damage sensitivity could have important implications for the choice of chemotheraphy treatment agents (Yarnold *et al*., 1993).

Conclusion

The finding of amplifications and deletions of regions in these three human gliomas cell lines by CGH and FISH before and after treatment with SMDB-Cd has opened up a new window for identifying subtypes of gliomas and may enable the identification of rational theraphies that address the fundamental molecular defects. This will hopefully be pursued further by other researchers so as to confirm and expand these primary findings.

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