Diaminobenzene Schiff Base Induces Caspase 9-dependent Apoptosis In U937 Leukemia Cells

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Abstract

Metal complex Schiff base compounds have been shown to be cytotoxic in vitro. However little is known concerning anticancer activity of Schiff bases that lack metal cations. The antineoplastic properties of two isomeric derivatives of diaminobenzene bis-Schiff base compounds, namely: \( N,N'\)-Bis(2-hydroxy-3-methoxybenzylidene)-1,2-diaminobenzene (2MJ) and \( N,N'\)-Bis(2-hydroxy-3-methoxybenzylidene)-1,3-diaminobenzene (2MH) towards U937 and K562 leukemia cell lines were investigated in this study. Both compounds were more cytotoxic towards U937 lymphoma cell with little effect on K562 cells when analysed using Sodium 3′-[1-(phenylamino-carbonyl)-3,4-Tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) cell proliferation assay technique. The IC\(_{50}\) value for 2MJ was almost half of 2MH. Both compounds were also found to induce apoptosis in U937 leukemia cells as evidenced by the induction of caspase 3 and 7. The level of caspase 3/7 induction was more pronounced in cells treated with 2MJ compared to 2MH. Caspase-9 was identified as the regulatory upstream caspase activated in U937 cells treated with 2MJ compared to 2MH. Caspase-9 was identified as the regulatory upstream caspase activated in U937 cells treated with 2MH and 2MJ, implicating the mitochondrial apoptotic pathway in diaminobenzene Schiff base-induced leukemia cell death. Both agents caused negligible effect on caspase-8 indicating a non Fas ligand receptor involvement in the apoptosis cascade. Neither compounds showed significant mutagenic outcome in the AMES mutagenicity assay. The result of this study highlights the potential of diaminobenzene bis-Schiff base compounds as a prospective agent to target cancer cells via the mitochondria.

Introduction

Anti-cancer agents target neoplastic cells and mainly cause their destruction via the apoptosis pathway. Apoptosis initiation may occur when there is direct DNA damage. Alternatively it could occur via adaptor proteins which can trigger the apoptosis cascade. Compounds that cause mitochondrial damage can lead to the release of cytochrome c which in turn may activate the apoptosis cascade (Martin and Forkert 2005) The increase in calcium concentration within a cell caused by drug activity via calcium binding protease calpain can also potentially trigger apoptosis. Other cause of apoptosis includes the involvement of apoptosis inducing factor (AIF) which is involved in initiating a caspase-independent pathway of apoptosis (positive intrinsic regulator of apoptosis) by causing DNA fragmentation and chromatin condensation. However the detail of their mode of action is still unclear. Schiff bases have wide biological activity antifungal (Jarrahpour, Khalili et al. 2007), antibacterial (R. NAIR 2006; F.M. Morad 2007; Hanna Hou 2007) and anticancer agents (J.D. Modi 1971; Tang Hongwu 1997). Even in industry they have been used as anticorrosive agents (Ashassi-Sorkhabi et al., 2005). Such wide applicability justifies the continued search for new Schiff base compounds. Metal complex of Schiff bases have been shown to cause significant anticancer activity (J.D. Modi 1971; Tang Hongwu 1997) They can cause free radical damage to the DNA of the cancer cells. The core structure of Schiff base compounds contain the functional group N=C, where the nitrogen atom is connected to an aryl or alkyl group, but not hydrogen, as shown in Figure (1). Although information concerning anticancer activity of metal complex Schiff bases are slowly trickling in, studies relating to the antineoplastic activity of Schiff bases that are devoid of metal cations are severely lacking. In this study we report the anticancer activity of two isomeric derivatives of a diaminobenzene Schiff base. The two bis-Schiff bases N, N′-bis-(2-hydroxy-3-methoxybenzylidene)-1,2-diaminobenzene (2MJ) and N, N′-bis-(2-hydroxy-3-methoxybenzylidene)-1,3-diaminobenzene (2MH), Figures (2) and (3), were synthesized as pure crystals (Al–Douh 2007; Al–Douh 2008a). The crystal structures for the two compounds are shown in Figures (4) and (5), respectively. The two compounds differ in their o-vanillin positions leading to a significant structural variation. 2MJ has its o-vanillin substructure located at the ortho position of its phenylenediamine unit, while 2MH has its substructure located at meta position of its phenylenediamine unit, as shown in Figures (2) and (3). The main objective of this work is to investigate the
anticancer activity of 2MJ and 2MH by utilizing U937 and K562 leukemia cells and determine the optimal structural configuration for a non-metallic Schiff base to give the most potent cytotoxic activity.

**Materials and Methods**

Dimethyl sulfoxide (DMSO) solvent, betulinic acid, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazoliium m-5-carboxanilide (XTT) and hoechst 33258 (hst) were all purchased from Sigma Co. Ltd, in a pure form. K562 and U937 leukemia cells were kindly donated by Dr. Tan Mei Lan from Universiti Sains Malaysia Advance Medical and Dental Institute (AMDI). RPMI-1641 medium was purchased from Life Technologies (California, USA).

Preparations of 2MJ and 2MH were described earlier (Al–Douh 2003a; Al-Douh 2003b; Al–Douh 2004). Caspase-3/7 and caspase-8 and caspase-9 detection kits were purchased from Promega (Promega, USA). Agents used for AMES assay were all obtained from Merck. MgSO4.7H2O, citric acid monohydrate, K2HPO4 and NaHNH4PO4.4H2O, glucose, agar and sodium azide were all purchased from Merck.

2.1 Cell proliferation assay

The cells were cultured in their respective growth medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Both K562 and U937 cell lines were cultured in RPMI-1641 medium with 10% fetal bovine serum and 1% of penicillin-streptomycin. The cells were seeded at 3 X 10^5 cells/ml and treated with the indicated compound concentrations, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg/ml. Each concentration was repeated in triplicate. After 48 hour treatment, XTT was added and incubated for 4 hrs. A Multiskan Ascent plate reader was used to record the absorbance reading at 450 nm.

2.2 Caspase activity assay

The apoptosis assay, employing the caspase kits, was conducted according to supplier's protocol (Promega, USA). Briefly, U937 cells were plated in a 96 well-plate at 5 X104 cell/ml and treated with 50 µg/ml of each compound in triplicate. Caspase-3/7 was prepared according to manufacturer's protocol by simply adding the provided 10 ml buffer to the powdered reagent. After 3 h, 100 µL of the Caspase-3/7 reagent was added to each concentration. After one hour, the luminescence emission spectra were measured for the samples. Betulinic acid was used as a positive control at 50 µg/ml concentration and DMSO was used as a negative control. Betulinic acid was used as a control because it has good cytotoxic activity on U937 leukemia cell line with an IC50 equals to 5 µg/ml and is able to induce apoptosis (Urech, Scher et al. 2005). The luminescence spectra were also measured for the DMSO dissolved in the culture medium for U937 cells using the same Multiskan Ascent plate reader. This procedure was repeated for the caspases-8 and -9 detection assays kits.

2.3 Mutagenicity AMES test

The Mutagenicity test was performed according to Linus Pauling Institute protocol. Salmonella typhimurium TA100 was preincubated for 24 h and then cultured for 24 h in nutrient agar. Several solutions were prepared before the test started. Histidine-Biotin solution, Vogel-Bonner medium E, 40% glucose solution and top agar solution were all prepared as described by same reference. Concentrations of histidine and biotin were stabilized in all assays. DMSO was used as negative control, by use of duplicate plates for each dose-step. The number of colonies was counted using a colony counter.

The final compound concentration exposed to the cells was 100 µg/ml. This was carried out in triplicate DMSO and sodium azide were used as negative and positive control, respectively. The plates were left for 48 hr before counting the number of colonies using a colony counter.

**Results**

Cytotoxicity and apoptosis study results for 2MJ and 2MH are presented here. The cytotoxic activity was measured in terms of IC50 value. The IC50 is the drug concentration at which cell growth is reduced by half. Lower IC50 indicates stronger cytotoxicity of a compound. The graphs shown in Figure (6) and (7) represent the dose response curves for U937 and K562 for 2MH and 2MJ respectively. The list of the IC50 values of both compounds against the two cancer cell lines are presented in Table (1).

A good apoptotic induction activity was observed using both compounds on the U937 cell line. Figure (8) shows the luminescence intensity values for 2MJ, 2MH, betulinic acid, DMSO and media (alone as a blank). The chart shows a much higher luminescent intensity for 2MJ (40.1 X 105) than for 2MH (14.3 X 105).

Figure (9) presents the ability of both compounds to cause caspase 8 and 9 induction. The luminescence intensity represents the induction ability. The highest triggering activity was seen for 2MJ on caspase 9 with 8 X 105 intensity level. For either compound, the ability to induce caspase 9 was significantly higher (P < 0.001) than the ability to induce caspase 8. This
shows the ability preference of each compound to induce the intrinsic pathway. The results confirmed a negative mutagenesis for the two compounds. However, this was proved by bacterial growth observation. The compounds, DMSO and phosphate buffer were added to different Petri dishes. After 48 h it was noticed that very little growth was observed in Petri dishes which contained phosphate buffer, 2MJ or 2MH with 300, 290 and 270 bacterial colonies, respectively, when compared to sodium azide with 700 colonies (see Figure (10)).

**Discussion**

The results of this study reveal that both 2MJ and 2MH differs significantly in their level of cytotoxic activities towards the two leukemia cell lines. The data in Table (1) shows that the cytotoxic potency of 2MJ towards U973 exceeds by almost two fold when compared to 2MH. The finding was similar in the 2MJ and 2MH treated K562 leukemia cells where the IC50 value for 2MJ was more than half of the IC50 value of 2MH. Both 2MJ and 2MH cause lower cytotoxic response towards K562 leukemia cells when compared to their activity towards U973 cells overall (see Figures 6 and 7). The K562 leukemia cells have Bcr-Abl over expression making it more resistant to apoptosis induction irrespective to the inducer which explains the lack of activity by both compounds towards this cell type (McGahon, Brown et al. 1997).

Consistent results was observed when comparing the activity of 2MJ and 2MH towards the pro-apoptosis cysteine proteases caspase 3 and 7 whereby 2MJ superseded 2MH caspase induction ability by more than two fold (Figure 8). The ability of both compounds to induce apoptosis via the caspase 3 and 7 was significantly higher than the betulinic acid positive control. In neuroectodermal tumor cells betulinic acid–induced apoptosis follows caspase activation, mitochondrial membrane alterations and DNA fragmentation. Betulinic acid has also been shown to induces apoptosis by directly perturbing mitochondria, leading to cytochrome-c release, which in turn regulates the "downstream" caspase activation (Simone Fulda and Klaus-Michael Debatin)

On closer scrutiny of 2MJ and 2MH activity on the caspases particularly the caspase 9 and 8, of which the former is the marker for DNA and mitochondrial insult, and the latter signifies Fas ligand receptor induced apoptosis, the result reveals that both compounds caused significant induction of caspase 9 and not caspase 8. The level of caspase 9 activation by 2MJ is also significantly higher than 2MH (p>0.001) and the DMSO positive control.

The apoptotic response, which leads to caspase-9 activation, is mainly contributed by DNA damage and mitochondrial insult. Thus it can be postulated that 2MJ, and to a lesser extent 2MH, may hinder the leukemia cell survival by perturbing the mitochondrial functions. The result of the caspase-8 and -9 responses towards the exposure of 2MH and 2MJ highlights the ability of these compounds to induce intrinsic apoptosis pathway. Like most non-alkylating minor groove binders, the AMES test results show that 2MJ and 2MH did not cause any mutagenic effects on the DNA. It is thus anticipated that the apoptosis caused by these agents is more likely due to mitochondrial damage rather than DNA damage.

**Conclusion**

This study concludes that diaminobenzene schiff bases have moderate antileukemic activity and they can cause cell death via apoptosis by potentially targeting the mitochondria of the leukemia cells. The extent of cytotoxic response was dependent on the structural conformation of the compound. 2MJ shows better anticancer activity than 2MH overall. This may be due to its more compact structure which enables it to penetrate through the membrane pores of the mitochondria and into its periplasmic space and interact with its key metabolic function.

**References**


Figure Captions

Figure (1): General Schiff base chemical structure

Figure (2): N,N'-Bis-(2-hydroxy-3-methoxybenzylidene)-1,3-diaminobenzene (2MH)

Figure (3): N,N'-Bis-(2-hydroxy-3-methoxybenzylidene)-1,3-diaminobenzene (2MH)

Figure (4): The crystal structure of 2MJ and the atomic numbering. The dashed lines indicate intramolecular hydrogen bonds. Adapted from Al-Douh, 2007

Figure (5): The crystal structure of 2MH and the atomic numbering. The dashed lines indicate intramolecular hydrogen bonds. Adapted from Al-Douh, 2007

Figure (6a) Graph showing the increase in U937 growth inhibition in correspondence with the increase in 2MJ concentration

Figure (6b) Graph showing the increase in U937 growth inhibition in correspondence with the increase in 2MH concentration

Figure (7a) Graph showing the increase in K562 growth inhibition in correspondence with the increase in 2M concentration

Figure (7b) Graph showing the increase in K562 growth inhibition in correspondence with the increase in 2MH concentration

Figure (8) shows the luminescent intensities for 2MJ, 2MH, Betulinic acid and DMSO obtained after adding caspase 3/7

Figure (9): shows the ability for 2MJ and 2MH to induce caspases 8 and 9 measured by detecting luminescent intensity.

Figure (10): chart shows the difference between the numbers of bacterial colonies
Illustrations

Illustration 1

Figure (1): General Schiff base chemical structure

Illustration 2

Figure (2): N,N'-Bis-(2-hydroxy-3-methoxybenzylidene)-1, 2-diaminobenzene (2MJ)
Illustration 3

Figure (3): N, N'-Bis-(2-hydroxy-3-methoxybenzylidene)-1, 3-diaminobenzene (2MH)

Illustration 4

Figure (4): The crystal structure of 2MJ and the atomic numbering.
Illustration 5

Figure (5): The crystal structure of 2MH and the atomic numbering.

Illustration 6

Graph showing increase in U937 growth inhibition in correspondence with 2MJ concentration increase
Illustration 7

Graph showing increase in U937 growth inhibition in correspondence with 2MH concentration increase

Illustration 8

Graph showing increase in K562 growth inhibition in correspondence with 2MJ concentration increase
Illustration 9

Graph showing increase in K562 growth inhibition in correspondence with 2MH concentration increase

Illustration 10

The luminescent intensities for 2MJ, 2MH, Betulinic acid and DMSO obtained after adding caspase 3/7
Illustration 11

The ability for 2MJ and 2MH to induce caspases 8 and 9 measured by detecting luminescent intensity.

![Illustration 11](image)

Illustration 12

Chart shows the difference between the numbers of bacterial colonies

![Illustration 12](image)
Table (1): Summary for IC50s for K562 and U937 cell lines using both compounds 2MJ and 2MH.
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