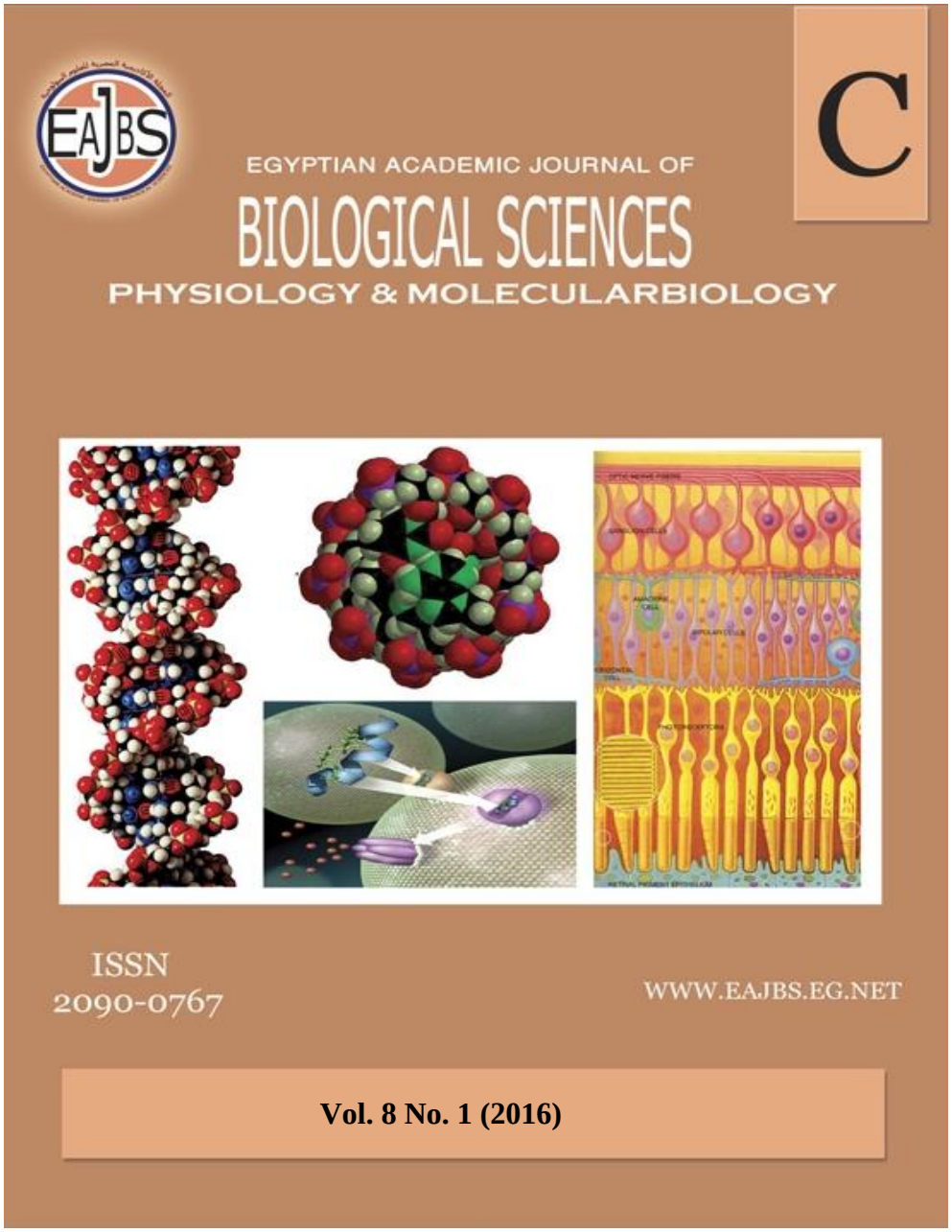

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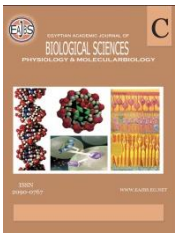

Augmentation of Curcumin Induced Cell Death by Cell Permeable C2 Ceramide in Human T-lymphoma Jurkat Cell Line

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ABSTRACT

Lymphoma in general is hard to treat cancer type which is resistant to be cured with variety of anticancer agents. Consequently, finding new approaches for anti-lymphoma agents is urgent. The present study evaluates the augmentation effect of cell permeable short chain C2 ceramide in sensitization of Human T-lymphoma Jurkat cells to curcumin-induced cell death. Many methods were used in the present study include MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide) assay for determination of cell viability, DAPI (4′,6-diamidino-2-phenylindole) and SRB (sulforhodamine B) methods for determination of cell death, Immunofluorescence staining of endogenous ceramide and western blot detection of apoptosis associated proteins. Curcumin alone reduces cell viability and induces apoptotic cell death in Jurkat cell line as indicated from cleavage of the proapoptotic caspase3 and cleavage of [poly (ADP-ribose) polymerase] PARP. C2 ceramide potentiates the apoptotic cell death induced by curcumin. Hence the combination of curcumin and C2 ceramide dramatically increases the apoptotic cell death associated proteins; cleaved caspase 3 and PARP in addition to apoptotic index obtained from DAPI and SRB assays. Moreover, the molecular mechanism of C2 ceramide sensitization of apoptotic cell death induced by curcumin was outlined.

INTRODUCTION

Curcumin [1,7-bis(4-hydroxy-3methoxyphenyl)-1,6- heptadien-3,5-dione] is a bioactive component isolated from turmeric. Curcumin has a broad spectrum of biological and chemotherapeutic properties as anti-inflammatory, antioxidant, anti-mutagenesis and anti-carcinogenesis (Azuine *et al*., 1992; Rao *et al*.,1995). Reviewing the available literature it was found that curcumin inhibited cellular proliferation and induced apoptosis in many leukemia cell line models (Piwocka *et al*., 2001; Blasius *et al*., 2006; Tan *et al*., 2006). It was found that curcumin induced a variety of biological affects in different leukemic cells as cell shrinkage, chromatin condensation, and DNA peroxidation (Sreejayan and Rao.,1994). Curcumin also induces activation of Bax and caspases, endoplasmic reticulum stress, and degradation of PARP in different leukemic cells (Sokoloski *et al*., 1997; Pan *et al*.,2001; Pae *et al*., 2007). Ceramide (Cer) is a sphingolipid which has a tumor suppressor properties and potent growth-suppressive effects in many cell types (Dbaibo*et al*., 1998).Ceramide is produced endogenously by at least two distinct ways.

First, the generation of Cer by hydrolysis pathway, this is triggered by the action of sphingomyelinases (SMases) which hydrolyze sphingomyelin (SM) to yield Cer and phosphorylcholine. There are three main groups of sphingomyelinases depending on their pH optimum: acidic, neutral or Alkaline with different cellular localizations (Kolesnick 2002; Ogretmen and Hannun 2004; Bartke and Hannun 2009). Second, endogenous Cer can be generated via the *de novo* synthesis pathway. In this pathway, serine and palmitoyl CoA are condensed by the action of serine palmitoyltransferase to form 3-ketosphinganine followed by reduction of the last to sphinganine which is then N-acylated by Cer synthases to produce dihydroceramide (Mandon *et al*. 1992). Short chain, cell-permeable ceramides include C2 and C6 Cer have shown cytotoxic effects against a variety of cancer cell lines (Radin *et al*., 1993) including Jurkat leukemia (Myrick *et al*., 1999). Many cell ligands and stressors were reported to induce Cer generation including TNF-α, Fas ligation, irradiation, heat shock (Yabu *et al*., 2008) and anti-cancer drugs (Jarvis and Grant, 1998) leading to the induction of apoptosis. It was also shown that curcumin induces Cer generation by different pathways depending on cell type (Moussavi *et al*., 2006; Kizhakkayil *et al*., 2012). Caspases are a family of cysteine-dependent aspartate-directed proteases that play a critical role in apoptosis. Ceramide generation triggers caspases activation in several apoptosis models (Watanabe *et al*., 2004; Dbaibo *et al*., 2007; Lafont *et al*., 2010). The current study investigates the role of C2 Cer in augmentation of curcumin induced apoptotic cell death induced in jurkat, human T-lymphoma, cell line. The data provides evidences that both curcumin and C2 Cer enhance the generation of endogenous Cer which in turn leads to caspase-dependent apoptosis and efficient cell death.

MATERIALS AND METHODS

Materials:

Curcumin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) were purchased from Sigma (USA). RPMI 1640 and fetal bovine serum (FBS) were from Lonza (Belgium). SuperSignal West Pico Chemiluminescent Substrate was from Pierce Biotechnology (USA). C2- ceramide was from Avanti (USA). Mouse anti-caspase 3 IgG, mouse anti PARP IgG, goat anti-mouse IgG-HRP, goat anti-β actin IgG and mouse anti-goat IgG-HRP were from Santa Cruz Biotechnology (USA). anti Cer IgM monoclonal MID15B4 from Alexis biochemicals, Lörrach (Germany) and goat anti-mouse IgM Alexa fluor 488 was from Invitrogen (USA). Protein Assay Kit from Bio-Rad (Austria). All other chemicals- if not mentioned- were purchased from Sigma co.

Cell culture and treatment:

Jurkat cell line was from ATCC. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS in 100% humidified atmosphere with 5% CO2 at 37°C. Twenty mM Stock solution of curcumin was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Cells were exposed to 10–20 μM curcumin or 5-20 μg/ml C2 Cer (in DMSO) or combination of both for up to 24 h. Control cells were grown in a medium containing equivalent amounts of the vehicle.

MTT assay:

After incubation of cells with drugs (2×10^3 cells/well in 96-well plates), growth medium was replaced with 200 μl of drug-free medium and supplemented with 50 μl of MTT (2 mg/ml) for 3 h. The formed crystals (Formazan) were dissolved in 200 μl of DMSO and quantified at 570 nm. The data are

expressed as the percentage of the viability of control cells exposed to DMSO alone.

DAPI staining :

Subconfluent cells were washed twice with serum free medium, treated with the indicated concentrations of curcumin and/or C2 Cer with or without 10 μM myriocin. Cells were reseeded at 2×10^5 cells/ml and incubated in 5% CO2 at 37°C. Then cells were washed and fixed with 1% glutraldehyde for 30 min, labeled with 2 μg/ml DAPI for 10 min. Cells were washed twice with PBS and examined for nuclear morphological changes under Leica fluorescent microscope.

SRB assay:

Cell toxicity determination using SRB method was performed according to (Vichai and Kirtikara, 2006) with small modifications. Briefly, after treatments, cells were fixed with 10% trichloro acetic acid and stained with SRB for 30 min, excess dye was removed by centrifugation (2.5 X 10^3 RPM for 3 min) and washing three times in 1% acetic acid then the bound dye is eluted in 10 mM Tris base and OD was determined at 510 nm using microplate reader.

Determination of apoptotic index:

Cells with morphological characters of apoptosis (early or late apoptosis from DAPI staining) was counted and the total number of cells was counted (about 1000 cells/experiment) in three independent experiments. The apoptotic index (AI) was calculated as the number of apoptotic cells expressed as percentage of total number of cells as previously described (Potten, 1996). *AI = no. of apoptotic cells/no. of total cells x100*. However SRB method does not distinguish between apoptotic or other cell death methods, but in the current investigation there is a strong correlation between AI from DAPI method and death index from SRB method. Also cell death induced by curcumin and or C2 Cer in the current study is morphologically and biochemically confirmed to be apoptotic cell death. So metaphorically, the death index calculated from SRB method as previously described (Vichai and Kirtikara, 2006) will be considered as apoptotic index. AI from SRB method was calculated as follow.

AI= 100 - OD of sample at final time/OD of sample at zero time x100.

Immunofluorescence detection of ceramide:

Cells were treated with 15μM curcumin or 10 μg/ml C2 ceramide or in combination with 10 μM myriocin for 12 h. Control cells were treated with the equivalent amount of DMSO. Cells were fixed with 2% formaldehyde for 10 min on ice and 20 min at room temperature. Then cells were cytospinned onto glass slides and permeablized with 0.1% Tx-100 for 10 min at room temperature. Blocking of active sites was done by 0.1% BSA in PBS for 30 min at room temperature. Anti Cer IgM mAb (MID15B4) was added according to manufacturers' recommendations for two h at room temperature followed by washing three times with 0.1% BSA in PBS. goat anti-mouse IgM Alexa fluor 488 was added according to manufacturers' recommendations for one h at room temperature. Washing three times with 0.1% BSA in PBS and mounting with mowiol medium. Examination and imaging were done using Leica fluorescence microscope.

Western blot:

After different drug treatments, cells were washed twice in cold PBS, pH 7.4 and lysed at 4°C in 300 μl of RIPA lysis buffer. Cells were disruption through 15-gauge syringe needle then cellular debris was removed by centrifugation (10,000 x g/ 10 min at 4°C). Protein concentration in the supernatant was determined and aliquots of 40 μg of total cellular lysates were subjected to electrophoresis on 10%

SDS-PAGE. Proteins were transferred to PVDF membrane using BioRad dry electro-transfer. blocking was done with 5% skim milk in TTBS (TBS with 0.05% Tween 20). Incubation with primary specific antibodies and HRP-conjugated secondary antibodies was carried out in blocking solution according to the manufacturers' instructions for 2 h at room temperature for the primary antibodies and 1 h at room temperature for the secondary antibodies. Visualization of Immunoreactive bands was done by SuperSignal Chemiluminescent kit. For equal loading confirmation, detection of β actin by anti-β actin IgG goat polyclonal antibody was used. The optical density of protein bands were estimated by Image J software as arbitrary units and then normalized to the density of the corresponding β actin bands.

Statistical analysis:

The statistical analysis was done using the Student's t test as the means ± SE, the significant values were taken as $p<0.05$.

RESULTS

C2 ceramide enhances Curcumin induced reduction of cell viability.

The effect of curcumin alone or in combination with C2 Cer on cell viability was investigated using MTT assay. As shown in Fig.1 A, cell viability is reduced gradually with increasing curcumin concentrations. The presence of 10 µg/ml of C2 Cer with curcumin significantly enhanced its cell viability reducing effect. However cell viability is reduced with increasing concentrations of C2 Cer (Fig. 1 B), but combination of 15 µM of curcumin with C2 Cer strongly potentiated its cell viability reduction. Also studying cell viability over 24h with each drug alone or in combination it was found that combination of both drugs significantly reduced cell viability compared to the effect of each drug alone, (Fig. 1 C).

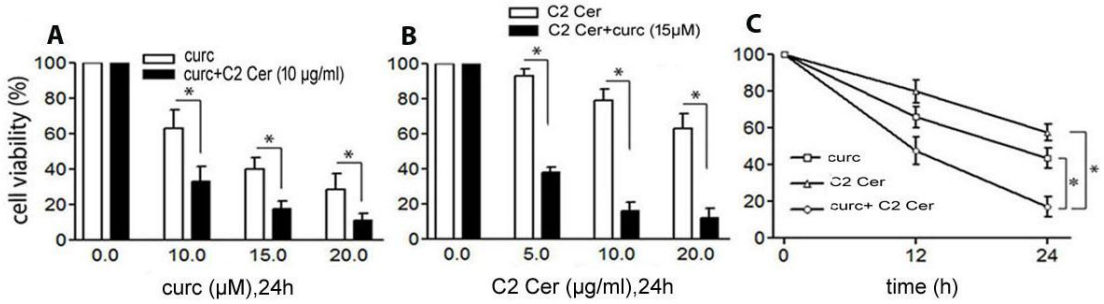

Fig.1: MTT assay for determination of cell viability after treatment of cells for 24 h with different concentrations of curcumin alone or in combination with 10 µg/ml of C2 Cer (A), different concentrations of C2 Cer alone or in combination with 15µM curcumin (B), and fixed concentrations of curcumin (15µM) or C2 Cer (10 µg/ml) or combination of both over 24h. Data are the mean ± SE from three independent experiments.*, significantly different at $p < 0.05$.

C2 Cer enhances curcumin induced-apoptotic cell death via caspase pathway.

Two different methods for detection of cell death were used, sulforhodamine B (SRB) method for cell toxicity screening and 4′,6-diamidino-2-phenylindole (DAPI) staining method for morphological detection of cell death. Apoptotic indices obtained after 24 h treatment with curcumin alone were 59.4 ± 5.4% and 56.7 ± 6.3%, and with C2 Cer alone were 36.2 ± 1.3% and 39.6 ± 2.6% and with curcumin plus C2 Cer were 83.6 ± 5.8% and 79.8 ± 4.6% as calculated from SRB and DAPI, respectively. The morphological characters of cell death induced by each drug alone or in combination seem to be apoptotic cell death as shown from DAPI

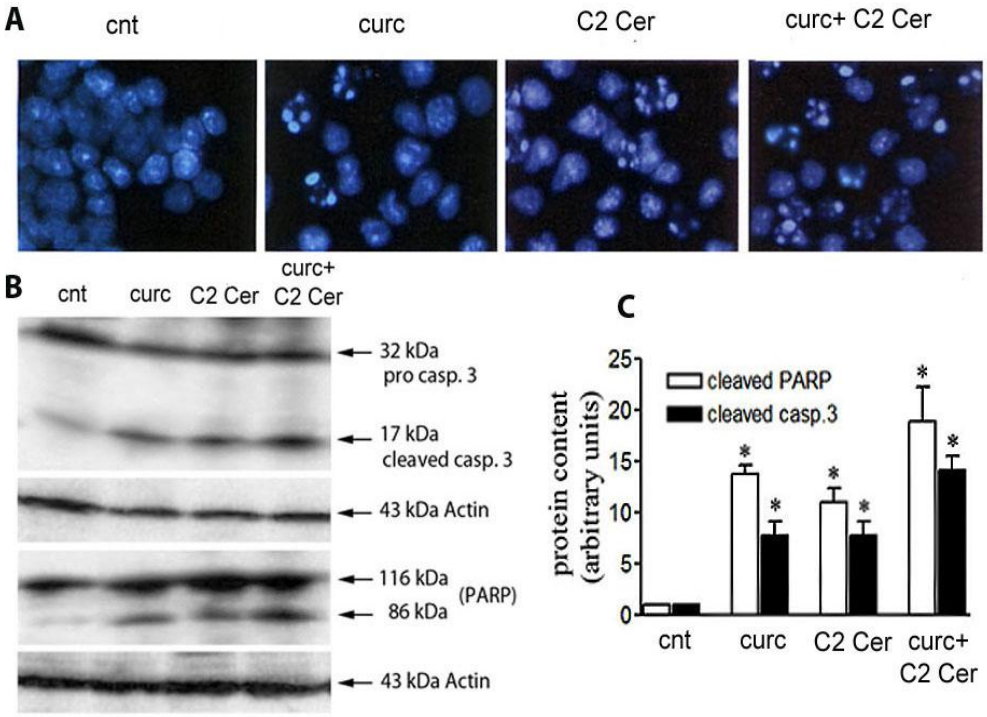

Fig. 2: Cells were treated with 15 µM curcumin or 10 µg/ml C2 Cer or both or the equivalent volume of DMSO as control for 24 h and then were stained with DAPI for examination of apoptotic cell death (A). Cells were treated as in (A) but for 6 h and then western blot detection of the indicated proteins was carried out (B), band density of Cleaved PARP and cleaved caspase3 from (B) was estimated by Image J software and then represented as arbitrary units after normalization to the corresponding value of Actin (C). Data are the mean ± SE from three independent experiments.*, significantly different at $p < 0.05$.

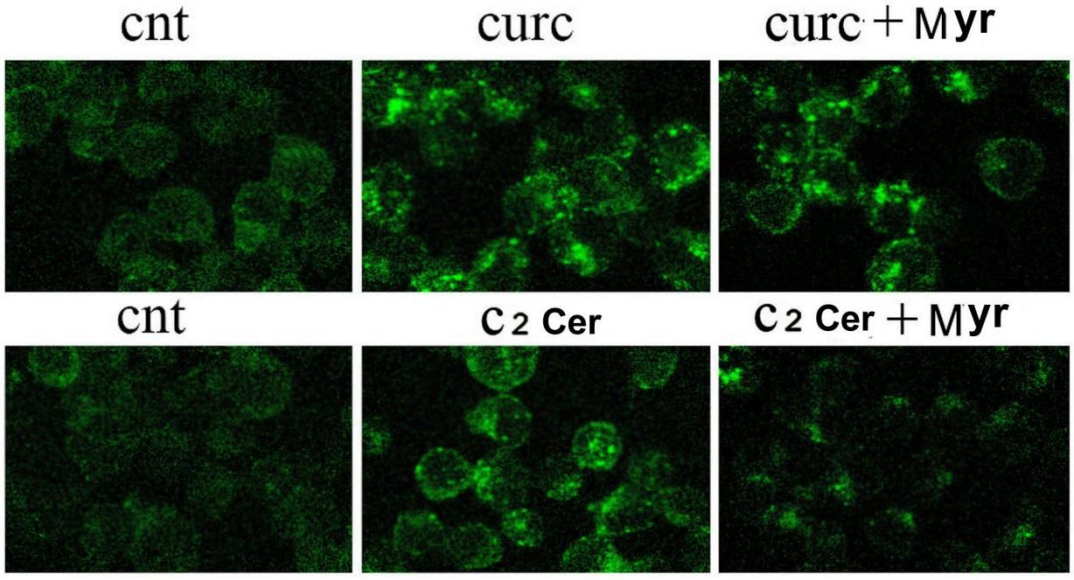

Fig. 3: Immunofluorescent staining of endogenous Cer after treatment of cells with 15 µM curcumin alone or in combination with 10 µM myriocin for 6h (upper panel), or with 10 µg/ml C2 Cer alone or in combination with 10 µg/ml myriocin for 6h (lower panel).Control cells were treated with the equivalent volume of DMSO.

staining images, (Fig. 2 A). In order to confirm the presence of apoptotic cell death, detection of cleaved caspase 3 and cleaved PARP were carried out by western blot. As shown in Fig. 2B, each drug alone activates caspase 3 and PARP cleavage and both drugs have higher cleavage effect on both proteins than each drug alone. Estimation of band density of cleaved caspase 3 and cleaved PARP was shown in (Fig. 2 C).

C2 ceramide enhances the de novo ceramide synthesis pathway.

Treatment of cells with cell permeable C2 Cer enhances the de novo ceramide synthesis (Takeda *et al*., 2006). Here, a potent de novo Cer synthesis inhibitor; Myriocin was used to test this possibility in jurkat cells. Ceramide was visualized by immuno- fluorescent staining using anti Cer IgM mAb antibody. It was found that either curcumin or C2 Cer alone evoked the production of endogenous Cer. The Cer produced by curcumin treatment was not inhibited by Myriocin but Myriocin reduced the endogenous Cer produced by C2 Cer treatment, (Fig. 3). This observation confirms the possibility of de novo Cer production by cell permeable C2 Cer treatment. Notice that Myriocin did not completely inhibited endogenous Cer production evoked by C2 Cer treatment, but there are some obvious Cer staining after myriocin treatment which indicates the presence of another pathway of Cer production evoked by C2 Cer in addition to de novo synthesis.

Myriocin has no effect on apoptotic cell death induced by curcumin and/or C2-ceramide.

Myriocin was used to test if Cer de novo synthesis pathway evoked by C2 Cer has any role in apoptotic cell death. It was found that Myriocin has no significant effect on the amount of PARP cleavage resulted from C2 Cer, curcumin or curcumin plus C2 Cer (Fig. 4 A). Accordingly, it seems that the role of C2 Cer in augmentation of cell death induced by curcumin is not based on the production of de novo Cer. Notably, curcumin alone did not evoke the production of de novo Cer synthesis but may be produce Cer from another pathway i.e. hydrolysis pathway.

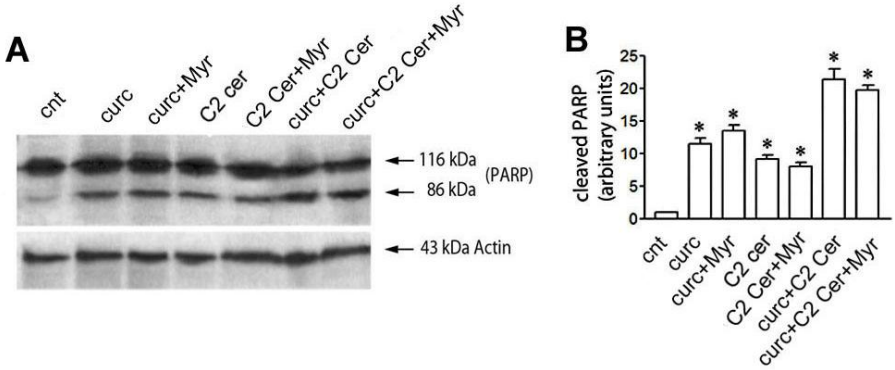

Fig. 4: Cells were treated with 15 µM curcumin and/or 10 µg/ml C2 Cer with or without 10 µM myriocin for 6 h, control cells were treated with the equivalent volume of DMSO, and then western blot detection of PARP and cleaved PARP was carried out (A). Optical density determination of the bands of cleaved PARP as calculated from (A) using ImageJ software and presented as arbitrary units after normalization with the corresponding Actin (B). Data are the mean ± SE from three independent experiments.*, significantly different at $p < 0.05$.

Cell viability was investigated after treatment with curcumin or C2 Cer in the presence of Myriocin. It was found that Myriocin could significantly enhance the cell viability when added to C2 Cer compared with C2 Cer alone, (Fig 5 A).Whereas, Myriocin has no significant effect on cell viability when combined with curcumin compared with curcumin alone, (Fig. 5, B).

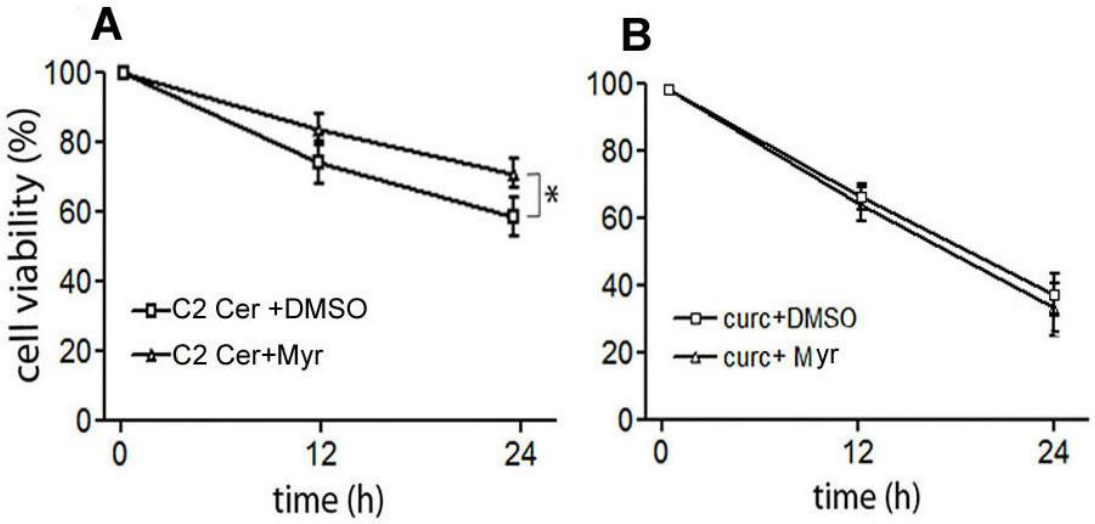

Fig. 5: Cell viability determination over 24 h by MTT assay after treatment of cells with 10 µM C2 Cer alone or in combination with 10 µM myriocin (A), or 15 µM curcumin alone or in combination with 10 µM myriocin (B). Data are the mean ± SE from three independent experiments.*, significantly different at $p < 0.05$.

Also apoptotic indices using SRB and DAPI methods give the same results. The apoptotic index for C2 Cer treatment is 37.3 ± 2.7% and 35.9 ± 1.3% as calculated from SRB and DAPI, respectively. Treatment of cells with C2 Cer in the presence of Myriocin resulted in a significant reduction of apoptotic index ($p< 0.5$), where it reaches 11.6 ± 0.9% and 9.4 ±0.4% as calculated from SRB and DAPI, respectively. With respect to curcumin treatment, Myriocin did not significantly change the apoptotic indices. Apoptotic index of curcumin alone is 62.5 ± 4.4% and 59.7 ± 5.8% and curcumin plus Myriocin is 63.3 ± 6.2% and 57.9 ± 8.6% as calculated from SRB and DAPI, respectively.

DISCUSSION

The data obtained from the current study show that C2 Cer potentiates lymphoma cells to curcumin- induced cell death. It was found that both C2 Cer and curcumin induce apoptotic cell death. C2 Cer promotes curcumin-induced caspase 3 and PARAP cleavage, so the mitochondrial apoptotic pathway is the most suggested cell death in this treatment. To confirm the data about the promotion effect of C2 Cer on cell death, some cell staining and viability assays were used as; MTT assay, SRB and DAPI methods. lymphomas in general are dangerous and hard to treat since they mainly affect the lymph nodes and may affect other organs and the bone marrow as well. Using of active ingredients of medicinal plants as complementary and alternative treatment, is an excellent and save source of chemopreventive and therapeutic agents for various human tumors (Fresco *et al*., 2010; Huang *et al*., 2010). One of the most famous used medicinal plants is turmeric. Curcumin, the principal curcuminoid of turmeric has been shown to inhibit proliferation and induce apoptosis in various types of solid tumor and leukemia cell lines (Tan *et al*., 2006). Cell-permeable short chain ceramides including C2 and C6 ceramides have anticancer effect against a variety of cancer cell lines (Auzenne *et al*., 1998; Myrick *et al*., 1999). Short chain Cer C6 was used to sensitize many cell lines for chemotherapy (Deshpande *et al*., 2008). However C2 Cer potentiates curcumine induced cell death but it is known that treatment of cells with exogenous cell permeable C2 Cer evokes

the production of endogenous Cer by de novo synthesis pathway (Takeda *et al*., 2006). Myriocin, was found to specifically inhibits the activity of serine palmitoyl transferase, (Miyake *et al*., 1995) the first enzyme in the de novo synthesis of Cer. Surprisingly, myriocin has no apparent effect on PARP cleavage induced by C2 Cer treatment. This indicates that de novo Cer yielded from C2 Cer treatment has no effect on apoptotic cell death resulted from C2 Cer. But when cell viability was investigated, myriocin was found to have a significant effect on cell viability compared with C2 Cer alone. This may according to the presence of another pathway of Cer production in addition to de novo pathway upon C2 Cer treatment. This assumption needs further investigation, this assumed pathway of Cer production is confirmed in the present investigation by the Immunofluorescence detection of Cer in cells treated by C2 Cer after inhibition of de novo pathway with myriocin (the anti Cer IgM monoclonal MID15B4 used in the present work does not recognize C2 Cer as stated by manufacturers). This also may clarify why there is no detectable effect of myriocin in the cleavage of PARP but in the same while myriocin reduces cell toxicity effect of C2 Cer. This suggests the presence of non-apoptotic cell death occurred by ceramide yielded from de no novo pathway.

In the present study, it was found that low dose of curcumin (10–15 μM) in the presence of C2 Cer can exceed the effect of the same concentration of curcumin alone. Also, C2 Cer with curcumin induce two different pathways of Cer generations which may be de novo and hydrolysis pathways which means more efficient cell death. It is clearly that C2 Cer sensitizes Jurkat cell line to curcumin-induced cell toxicity which means using lower concentration of curcumin in combination with C2 Cer.

The present research has provided a new approach concerning the treatment strategy for fighting lymphomas in addition to underlying the molecular mechanism of C2 Cer cytotoxic effect which need further investigation for its validation as chemo-preventative and/or chemo-therapeutic agent against lymphoma.

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ARABIC SUMMERY

زيادة الموت الخلوي المستحث بواسطة الكركومين عن طريق السيراميد ثنائي الكربون النافذ للخلية في خط خلايا السرطان البشرية اللمفية التائية جوركات

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سرطان الخلايا اللمفية عموما من أنواع السرطانات صعبة المعالجة حيث أنها مقاومة للعلاج باستخدام العديد من العوامل المضادة للسرطان. لذلك يعد البحث عن عوامل جديدة مضادة لسرطان الخلايا اللمفية من الأمور الحتمية. البحث الحالي يقيم زيادة حساسية خلايا السرطان البشرية اللمفية التائية جوركات للموت الخلوي المستحث بواسطة الكركومين باستخدام السيراميد قصير السلسلة الكربونية النافذ للخلية. استخدم في البحث الحالي الكثير من الطرق البحثية منها طريقة MTT لدراسة حيوية الخلايا و طرق SRB, DAPI لدراسة الموت الخلوي. واستخدم أيضا الكشف المناعي الفلورى للكشف عن السيراميد الداخلي للخلية وكذلك استخدم الكشف المناعي للبروتين لتحديد البروتينات المرتبطة بالموت الخلوي المبرمج مثل انشطار الكاسباز ٣ والبارب. ولقد وجد أن السيراميد قصير السلسلة الكربونية يحفز الموت الخلوي المبرمج الناتج من معالجة الخلايا بالكوركومين. حيث أن معالجة الخلايا بالكوركومين والسيراميد أدى إلى الحصول على نسبة عالية من البروتينات المرتبطة بالموت الخلوي المبرمج المذكورة عاليه. بالإضافة إلى زيادة معامل الموت الخلوي المبرمج الذي تم حسابه من طرقSDB, DAPI . بالإضافة إلى انه تم توضيح الميكانيكية الجزيئية التي يعمل بها السيراميد قصير السلسلة الكربونية في تحفيز الموت الخلوي المبرمج المستحث بواسطة الكركومين.