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Full Communication

Anti-Obesity Effects of Xanthohumol Plus Guggulsterone in 3T3-L1 Adipocytes

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ABSTRACT Xanthohumol (XN) and guggulsterone (GS) have each been shown to inhibit adipogenesis and induce apoptosis in adipocytes. In the present study effects of the combination of XN + GS on 3T3-L1 adipocyte apoptosis and adipogenesis were investigated. Mature adipocytes were treated with XN and GS individually and in combination. XN and GS individually decreased cell viability, but XN + GS caused an enhanced decrease in viability and potentiated induction of apoptosis. Likewise, XN + GS caused a potentiated increase in caspase-3/7 activation, whereas neither of the compounds showed any effect individually. In addition, western blot analysis revealed that XN + GS increased Bax expression and decreased Bcl-2 expression, whereas individual compounds did not show any significant effect. XN and GS both decreased lipid accumulation. Individually, XN at 1.5 μM and GS at 3.12 μM decreased lipid accumulation by $26 \pm 4.5\%$ ($P < .001$) each, whereas XN1.5 + GS3.12 decreased lipid accumulation by $78.2 \pm 1.8\%$ ($P < .001$). Moreover, expression of the adipocyte-specific proteins was down-regulated with XN1.5 + GS3.12, but no effect was observed with the individual compounds. Finally, XN + GS caused an enhanced stimulation of lipolysis. Thus, combination of XN and GS is more potent in exerting anti-obesity effects than additive effects of the individual compounds.

KEY WORDS: • ***adipogenesis*** • ***apoptosis*** • ***Bcl-2 family proteins*** • ***caspase-3/7*** • ***peroxisome proliferator-activated receptor*** γ

INTRODUCTION

OBESITY IS AMONG THE MOST easy to recognize and the most difficult to treat of medical conditions. It is a chronic metabolic disorder that results from the imbalance between energy intake and energy expenditure. When energy intake is less than output, mobilization of triglycerides leads to decrease in adipose tissue mass. Although adipocyte number generally does not decrease with fat mobilization, under some conditions decrease in adipocyte number can occur from preadipocyte and mature adipocyte apoptosis.[1] Thus, inhibition of adipogenesis, adipocyte apoptosis, lipolysis, and fatty acid oxidation are important mechanisms involved in reducing body fat. We have previously demonstrated that combinations of natural products like genistein and resveratrol are more effective than individual compounds alone in inhibiting adipogenesis and inducing apoptosis and lipolysis in adipocytes under *in vitro* conditions.[2,3] In the current study we investigated whether the combination of xanthohumol (XN) and guggulsterone (GS) would result in potentiated effects on adipocyte apoptosis and adipogenesis.

XN is a flavonoid found in the hop plant, *Humulus lupulus* L. Hops are used to add bitterness and flavor to beer, and therefore the main dietary source of XN is beer. XN is well known for its anticancer[4] and antioxidant properties. XN was also reported to exert inhibitory effects on the synthesis of triglycerides and the expression of diacylglycerol acyltransferase-1 (DGAT1) and other adipocyte-specific transcription factors in 3T3-L1 adipocytes.[5] In addition, XN was shown to inhibit cell proliferation and induce apoptosis in adipocytes[5] and several cancer cell lines.[6,7] GS, the active substance in guggulipid, has been of interest because of its effects on lipid metabolism: guggulipid has been found to reduce triglyceride levels as well as cholesterol levels.[8] In one study, oral administration of gum guggul alone was shown to decrease body weight in both animals and humans.[9] More recent studies have confirmed the effect on cholesterol levels and have shown that this activity is at least partly due to the antagonism of nuclear farnesoid X receptors by GS.[10,11] Specific effects of GS on decreasing lipid accumulation and inducing apoptosis in 3T3-L1 adipocytes has been reported recently.[12]

The aim of the current study was to examine the combination effects of XN + GS on inducing apoptosis, inhibiting lipid accumulation and stimulating lipolysis in the well-characterized mouse 3T3-L1 preadipocyte cell line. We show that the combination of XN + GS caused an enhanced apoptosis in mature adipocytes through the mitochondrial

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death pathway by altering the expression of Bax and Bcl-2 proteins with subsequent activation of caspase-3/7. Furthermore, in mature adipocytes the combination of XN + GS also stimulated lipolysis. In maturing preadipocytes, XN + GS decreased the expression of adipocyte-specific transcription factors more than either compound alone, resulting in decreased lipid accumulation.

MATERIALS AND METHODS

Cell line and cell culture

3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described elsewhere.[13] In brief, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY) containing 10% bovine calf serum until confluent. Two days after confluency (D0), the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS), 167 n*M* insulin, 0.5 μ*M* 3-isobutyl-1-methylxanthine, and 1 μ*M* dexamethasone for 2 days (D2). Cells were then maintained in 10% FBS/DMEM with 167 n*M* insulin for another 2 days (D4), followed by culturing with 10% FBS/DMEM for an additional 4 days (D8), at which time more than 90% of cells were mature adipocytes with accumulated fat droplets. All media contained 100 U/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL glutamine (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cell viability assay

Adipocytes were incubated with dimethyl sulfoxide (DMSO) or test compounds. Prior to measuring viability, treatment media were removed and replaced with 100 μL of fresh 10% FBS/DMEM and 20 μL of 3-(4,5-dimethythizol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) solution. Cells were then returned to the incubator for 1 hour, and the absorbance was measured at 490 nm in a plate reader (μQuant™, Bio-Tek Instruments, Inc., Winooski, VT) to determine the formazan concentration, which is proportional to the number of live cells.

Apoptosis assay

For the assessment of apoptosis, the ApoStrand™ ELISA Apoptosis Detection Kit (BIOMOL International, Plymouth Meeting, PA) was used. This kit detects single-stranded DNA, which occurs in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis.[14] Adipocytes were incubated with DMSO or test compounds for the times and concentrations given in Results and figure legends. Thereafter, treatment medium was removed, and the cells were fixed for 30 minutes and assayed according to the manufacturer's instructions. For Hoechst staining, cells treated with either 0.2% DMSO or test compounds were fixed with 3.7% formaldehyde in phosphate-buffered saline (GIBCO) and incubated for 15–30 minutes with 5 μg/mL Hoechst 33342. The nuclear staining was examined under a fluorescence microscope.

Caspase-3 and -7 activity assay

Adipocytes were incubated with DMSO or test compounds. Thereafter, 100 μL of Caspase-Glo™ 3/7 reagent (Promega, Madison, WI) was added to each sample, and the cells were incubated for 1 hour and assayed according to the manufacturer's instructions.

Quantification of lipid content

Lipid content was quantified using commercially available AdipoRed™ Assay Reagent (Cambrex Bioscience, Walkersville, MD) according to the manufacturer's instructions. In brief, for the experiment shown in Figure 4, test compounds along with DMSO control were added with the induction medium for days 0–6 of adipogenesis. On day 6, intracellular lipid content was measured by the AdipoRed assay. Cells were washed with phosphate-buffered saline, and 200 μL of phosphate-buffered saline was added to the wells. AdipoRed reagent (5 μL) was added to each well. After 10 minutes, fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 572 nm. In a separate experiment, treated cells were stained with Oil Red O and hematoxylin on day 6 to visualize lipid content as described by Suryawan and Hu.[15]

Lipolysis assay

Adipocytes were treated with DMSO or test compounds for 12 hours. At the end of the incubation, 100 μL of the conditioned medium was removed and transferred to the corresponding well of the new plate. One hundred microliters of glycerol reagent (Zen-Bio, Research Triangle Park, NC) was added, and cells were incubated at room temperature for 15 minutes. The absorbance was measured at 540 nm in a μQuant plate reader to determine the glycerol content of the sample.

Western blot analysis

Mature adipocytes were treated with either DMSO or test compounds for 24 hours. Likewise, maturing preadipocytes were treated with either DMSO or test compounds for 6 days during the stage of differentiation. Whole-cell extracts were prepared as described elsewhere.[16] The protein concentration was determined by bicinchoninic assay with bovine serum albumin as the standard. Western blot analysis was performed using the commercial NUPAGE® system (Novex/Invitrogen), in which a lithium dodecyl sulfate sample buffer (Tris/glycerol buffer, pH 8.5) was mixed with fresh dithiothreitol and added to samples. Samples were then heated to 70°C for 10 minutes, separated by 12% acrylamide gels, and analyzed by immunoblotting as previously described.[17]

Quantitative analysis of western blot data

Measurement of signal intensity on polyvinylidine membranes after western blotting with various antibodies was performed using a FluorChem densitometer with the AlphaEaseFC image processing and analysis software (Alpha Innotech Corp., San Leandro, CA). For statistical analysis, all data were expressed as integrated density values, which were calculated as the density values of the specific protein bands/β-actin density values and expressed as a percentage of the control. All figures showing quantitative analysis include data from at least three independent experiments.

Statistical analysis

One-way analysis of variance (GLM procedure, Statistica, version 6.1; StatSoft, Inc., Tulsa, OK) was used to determine significance of treatment effects. Fisher's *post hoc* least significant difference test was used to determine significance of differences among means. In some cases in order to estimate differences between the combined treatments and a hypothetical additive treatment response, a sum of the individual treatment effects for each replicate was calculated, and these numbers were included in the analysis of variance. Statistically significant differences are defined at the 95% confidence interval. Data shown are mean $\pm$ SEM values.

Reagents

XN and GS ($\geq$98%) were purchased from Sigma (St. Louis, MO). The viability assay kit (CellTiter 96® Aqueous One Solution Cell Proliferation Assay; containing MTS assay reagent) was purchased from Promega. Antibodies specific for Bax, Bcl-2, β-actin, peroxisome proliferator-activated receptor (PPAR) γ, CCAAT/enhancer binding protein (C/EBP) α, aP2, and DGAT1 were from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

XN + GS decreased cell viability and induced apoptosis

Mature 3T3-L1 adipocytes were treated with XN (12.5 or 25 μM) and GS (50 or 75 μM) as individual compounds and in combinations for 48 hours. The cell viability data indicated that both XN and GS decreased cell viability, but the combination of XN + GS was more potent than either compound alone and was more potent than the additive

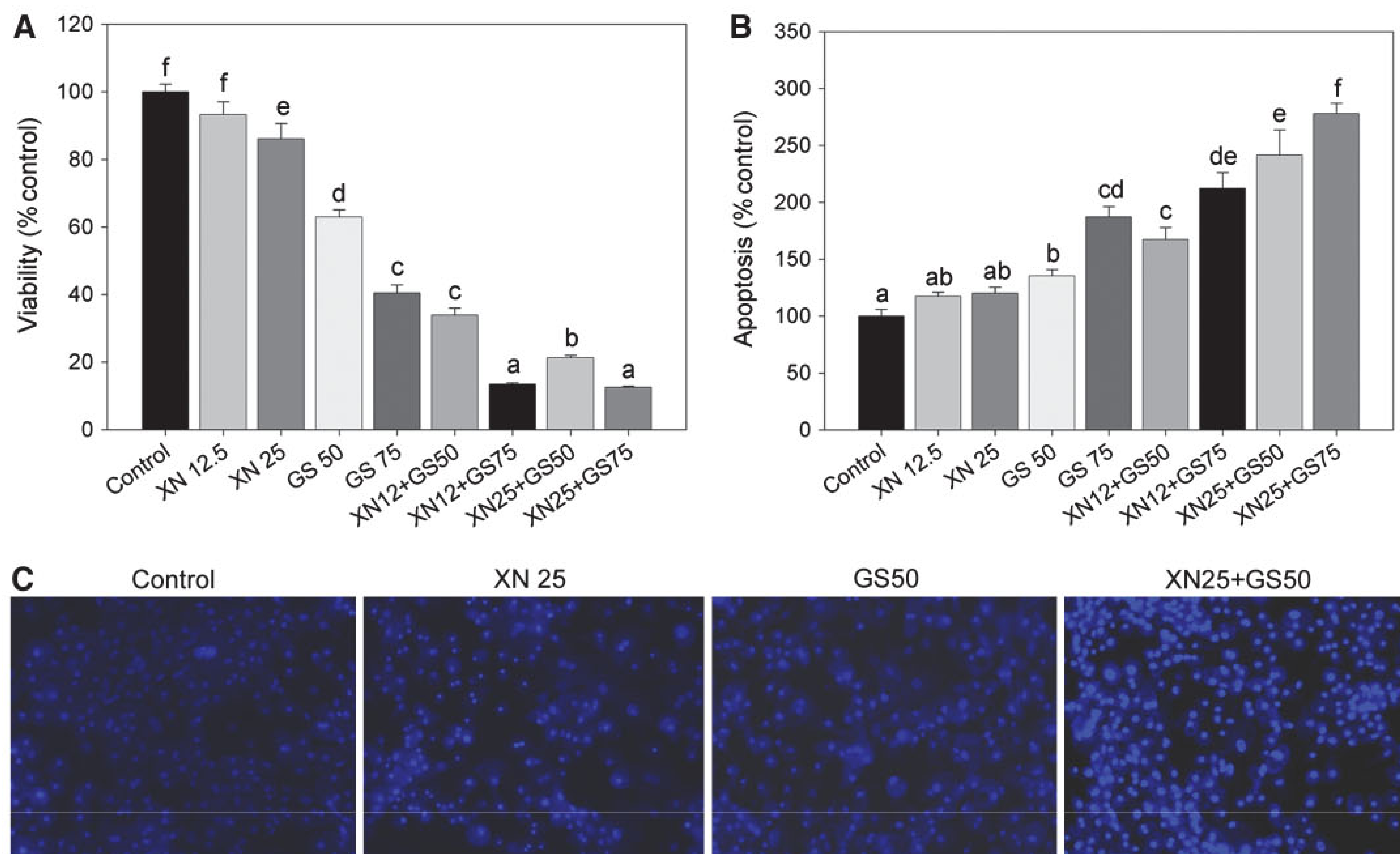


FIG. 1. Effect of XN and GS on mature adipocyte viability and apoptosis. 3T3-L1 mature adipocytes were treated with either 0.2% DMSO (control) or test compounds at various concentrations for 48 hours. Cell (**A**) viability and (**B**) apoptosis were determined as described in Materials and Methods and Results. The experiment was repeated two times. Data are mean $\pm$ SEM (vertical bars) values ($n = 8$). Means not designated by a common superscript are different ($P < .001$). (**C**) Representative images of Hoechst staining.

effect of the individual compounds. XN25 and GS50 as single compounds decreased cell viability by 14.0 ± 4.54% and 37.0 ± 2.09%, respectively ($P < .001$) (Fig. 1A). XN25 + GS50 decreased cell viability by 78.7 ± 0.68%, whereas the calculated additive response would have been only a decrease of 50.9 ± 3.7% ($P < .001$) (Table 1). Likewise, GS50 increased apoptosis 35.1 ± 5.8% ($P < .05$) more than the control, although XN25 did not have a significant effect; XN25 + GS50 increased apoptosis by 141.6 ± 22.4% more than the control ($P < .001$) (Fig. 1B). The calculated additive response, however, would have been an increase of only 55 ± 8.6% ($P < .001$), which is significantly less than the combined effect (Table 1). An increase in apoptotic nuclei with combination treatment was also visualized with Hoechst staining (Fig. 1C).

XN + GS induced caspase-3/7 activation

To confirm apoptosis in response to the treatment with XN + GS, we evaluated caspase-3/7 activity. Adipocytes were treated for 24 hours with XN (12.5 or 25 μM) and GS (50 or 75 μM) as individual compounds or in combination. Both XN and GS increased caspase-3/7 activity (Fig. 2). XN25 and GS50 had no significant effect on caspase-3/7 activation, whereas XN25 + GS50 increased caspase-3/7 activation by 65.6 ± 10.7% ($P < .001$), which is significantly more than the calculated additive response (Table 1). XN25 and GS50 were selected for subsequent western blotting experiments.

XN + GS altered the expression of Bcl-2 family proteins

To investigate the involvement of the Bcl-2 family in XN + GS-induced apoptosis, we measured the cellular levels of Bcl-2 and Bax in adipocytes treated with XN25, GS50, and XN25 + GS50 by western blotting. The expression of Bax was increased by 81.3 ± 10.6% ($P < .001$) with XN25 + GS50, whereas individual compounds did not significantly alter Bax expression. Likewise, Bcl-2 expression was decreased by 50.6 ± 7.8% ($P < .001$) with XN25 + GS50, whereas individual compounds showed no significant effect (Fig. 3).

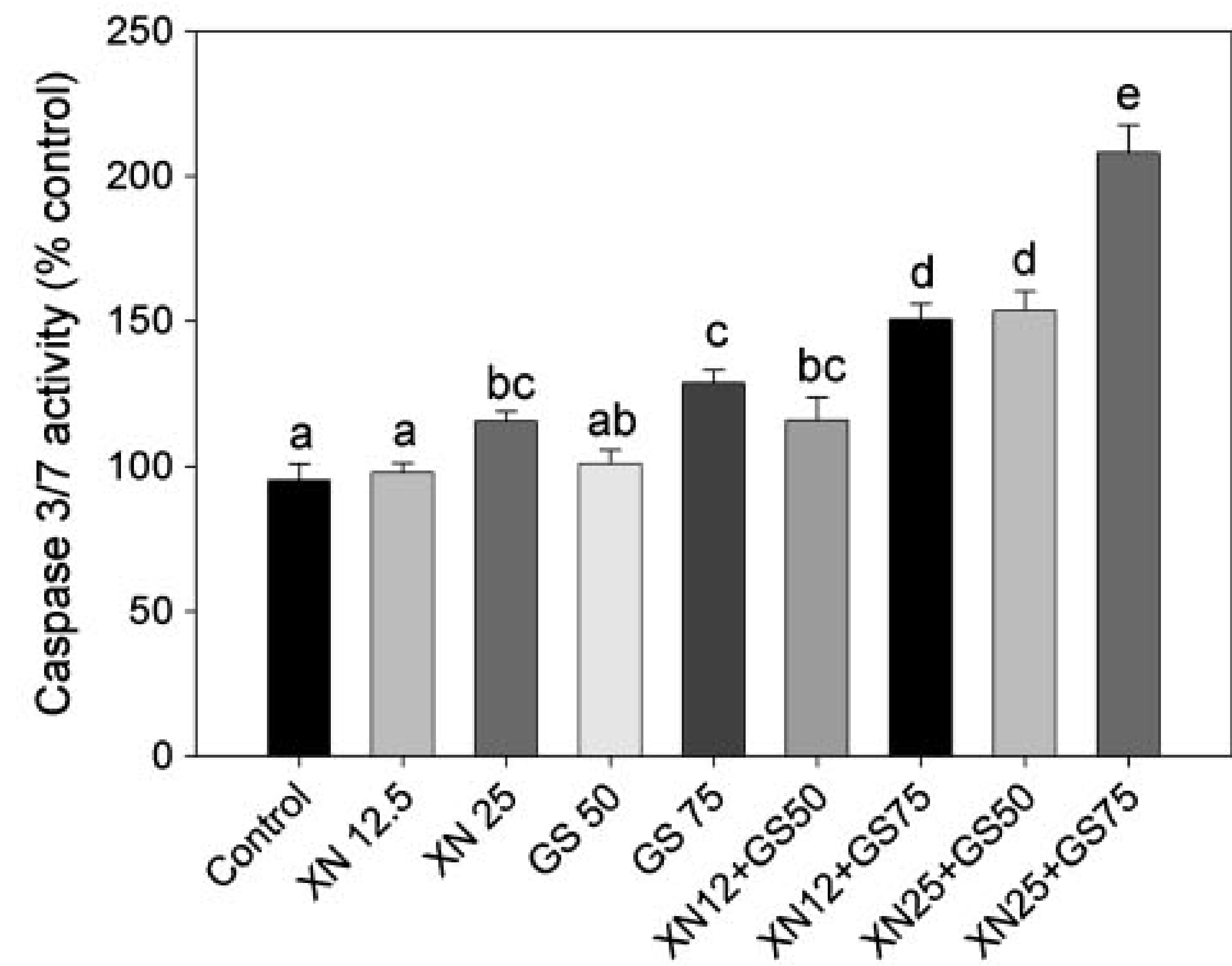


FIG. 2. Effect of XN and GS on caspase-3/7 activity. 3T3-L1 mature adipocytes were treated with either 0.2% DMSO (control) or test compounds for 24 hours as described in Results. Caspase-3/7 activity was measured following the manufacturer's (Promega's) instructions. Data are mean ± SEM (vertical bars) values ($n = 8$). Means not designated by a common superscript are different ($P < .001$).

XN + GS inhibited lipid accumulation

In maturing preadipocytes, preliminary experiments with a range of XN and GS concentrations (data not included) showed that the combined effect on adipogenesis was very potent. Therefore, lower concentrations were used in experiments with maturing preadipocytes. The results showed that XN and GS as individual compounds decreased lipid accumulation (Fig. 4A). However, no significant effect on cell viability was observed. XN1.5 and GS3.12 decreased adipogenesis by about 26.0 ± 4.5% ($P < .001$) each, whereas XN1.5 + GS3.12 decreased lipid accumulation by 78.2 ± 1.8% ($P < .001$). The calculated additive response of XN1.5 + GS3.12 would have been a decrease in lipid accumulation by 53.1 ± 5.8% ($P < .001$). Similar results were observed using Oil Red O staining to visualize lipid accumulation in cells after treatments (Fig. 4B). XN1.5 and GS3.12 were selected for subsequent western blotting experiments.

TABLE 1. PERCENTAGE CHANGE IN VIABILITY, APOPTOSIS, CASPASE-3/7 ACTIVATION, AND LIPOLYSIS IN 3T3-L1 MATURE ADIPOCYTES TREATED WITH 25 μM XN (XN25) PLUS 50 μM GS (GS50)

	(% change)			
Treatment	*Cell viability at 48 hours*	*Single-stranded DNA at 48 hours*	*Caspase-3/7 activity at 24 hours*	*Lipolysis at 12 hours*
Control (0.2% DMSO)	0.0 ± 2.2^{a}	0.0 ± 5.9^{a}	0.0 ± 5.5^{a}	0.0 ± 0.96^{a}
XN25	13.9 ± 4.5^{b}	19.6 ± 5.5^{ab}	15.2 ± 6.6^{a}	13.2 ± 4.6^{b}
GS50	37.0 ± 2.0^{c}	35.5 ± 5.8^{bc}	9.81 ± 10.6^{a}	21.5 ± 5.0^{bc}
XN25 + GS50	78.7 ± 0.6^{e}	141.6 ± 22.1^{d}	65.6 ± 10.7^{b}	44.2 ± 2.4^{de}
XN25GS50 (calculated additive response)	50.9 ± 3.7^{d}	55.0 ± 8.6^{c}	25.0 ± 10.1^{a}	34.7 ± 9.7^{cd}

Values not designated by a common superscript are different ($P < .001$).

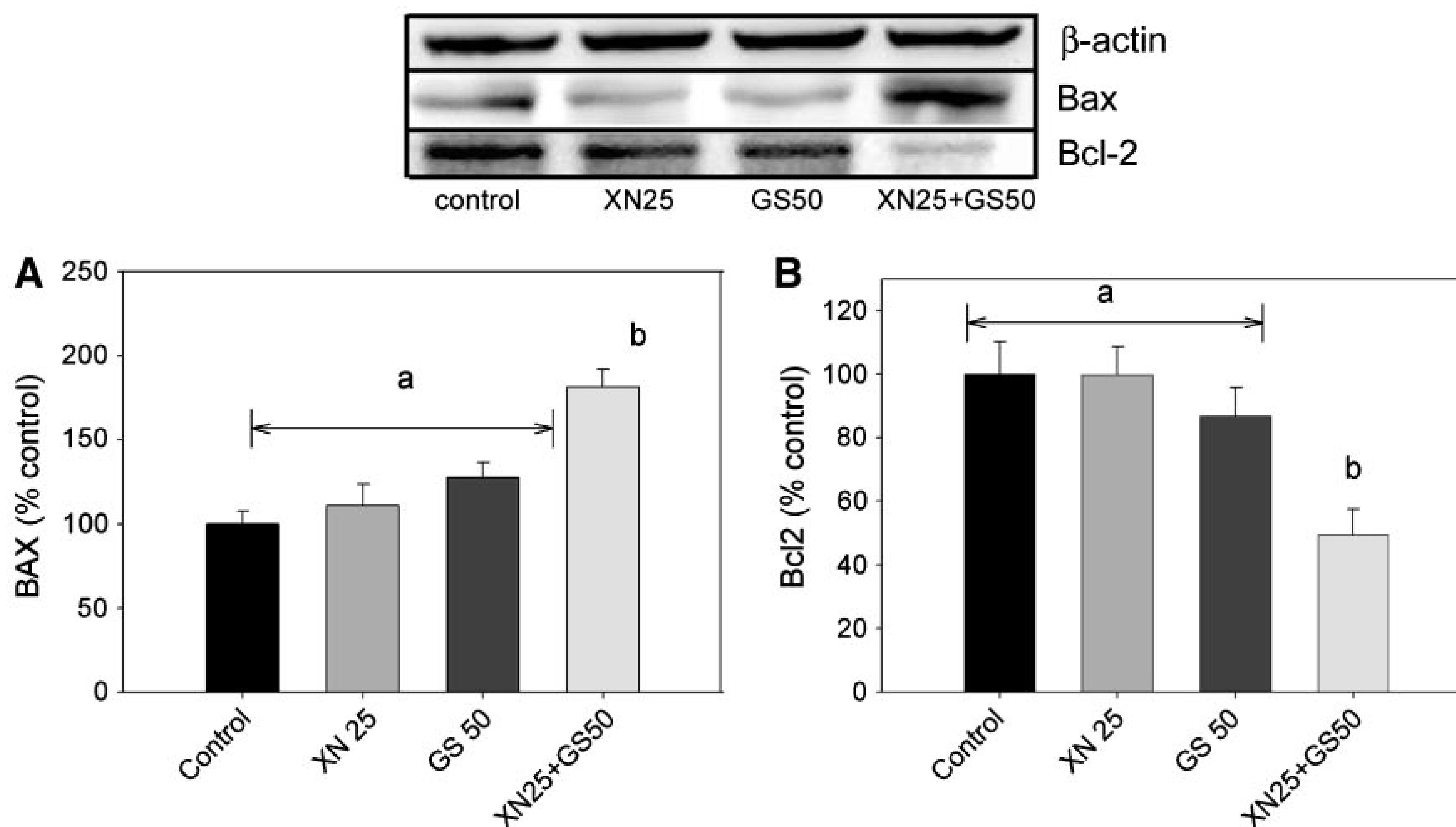
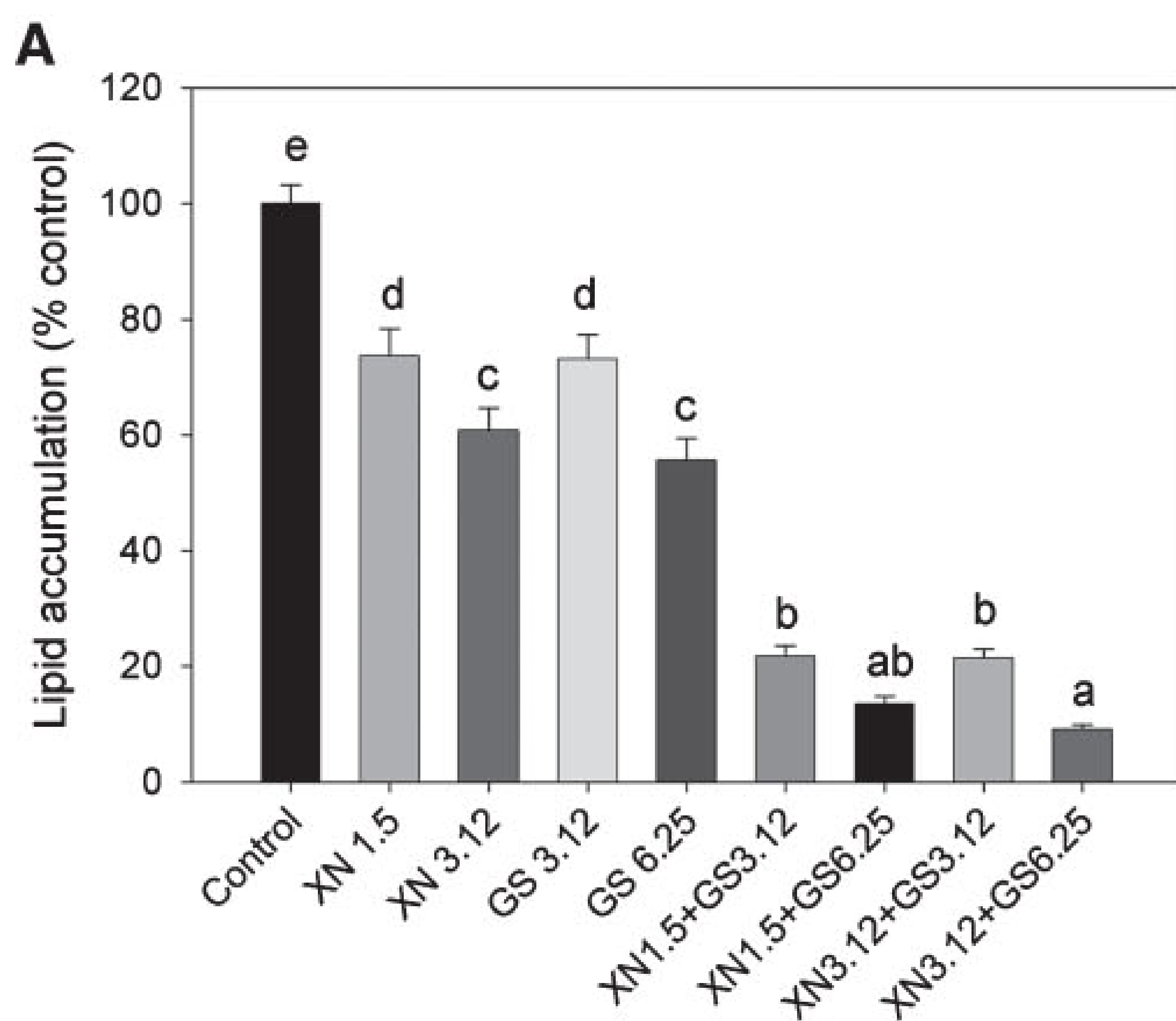


FIG. 3. Effect of XN and GS on expression of Bax and Bcl-2. On day 6 after treatment whole-cell lysates were extracted and subjected to western blotting as described in Materials and Methods. Densitometric quantitation of the autoradiograms for all the protein bands were performed, and integrated density values of (**A**) Bax and (**B**) Bcl-2 were calculated and expressed as percentages of the control. Data are mean ± SEM (vertical bars) values ($n = 3$). Means not designated by a common superscript are different ($P < .001$).

XN + GS decreased PPARγ, C/EBPα, aP2, and DGAT1 expression

To determine whether the decrease in lipid accumulation with XN and GS was related to changes in PPARγ, C/EBPα, aP2, and DGAT1 expression levels, 3T3-L1 cells were treated with either DMSO or test compounds from 0 to 6 days in the differentiation process. On day 6 whole-cell lysates were prepared as described previously and subjected to western blotting. Quantitative analysis revealed that XN and GS did not alter the expression of adipocyte-specific genes as individual compounds, but XN1.5 + GS3.12 decreased the expression levels of PPARγ, C/EBPα, aP2, and DGAT1 by 51.6 ± 6.4%, 46.1 ± 6.4%, 49.7 ± 7.1%, and 46.2 ± 8.8% ($P < .001$), respectively (Fig. 5).



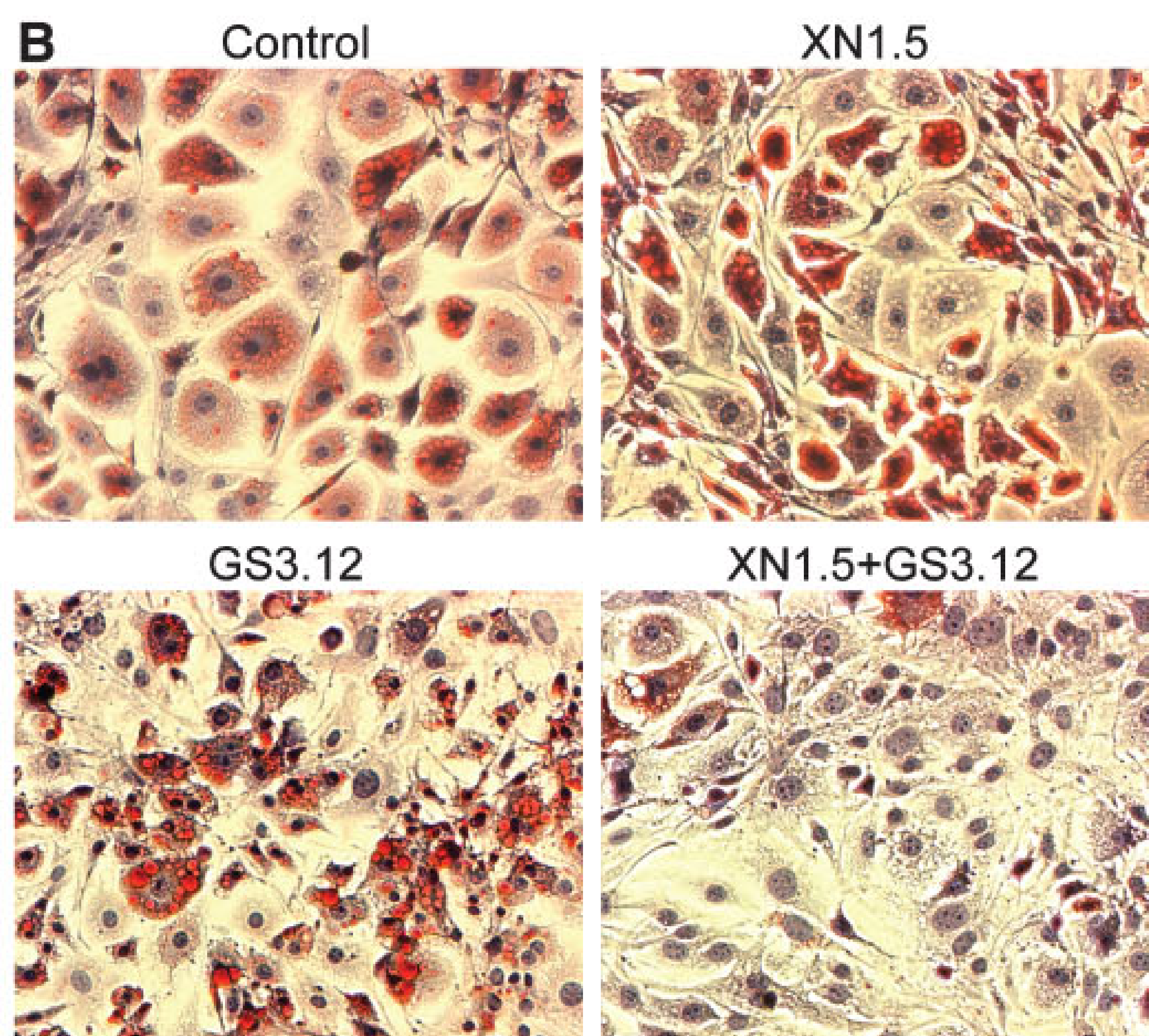


FIG. 4. Effect of XN and GS on lipid content of 3T3-L1 maturing preadipocytes. (**A**) Lipid content measured by AdipoRed assay. Cells were treated with either 0.2% DMSO (control) or test compounds. The experiment was repeated twice. Data are mean ± SEM (vertical bars) values ($n = 6$). Means not designated by a common letter are different ($P < .001$). (**B**) Representative images of Oil Red O staining.

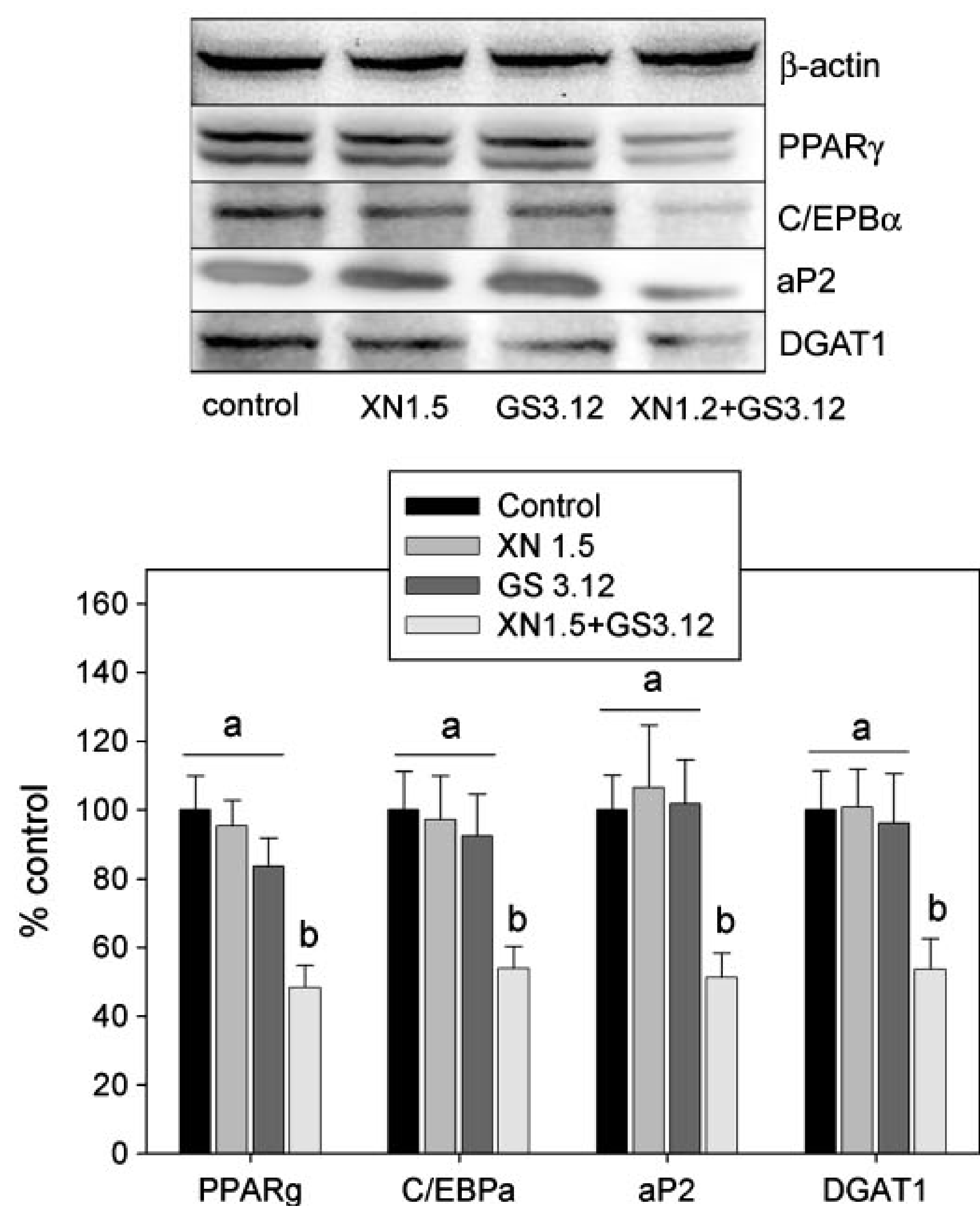


FIG. 5. Effect of XN and GS on PPARγ, C/EBPα, aP2, and DGAT1 expression in maturing 3T3-L1 preadipocytes. Whole-cell lysates were extracted on day 6 after treatment and subjected to western blotting. Data are mean ± SEM (vertical bars) values ($n = 3$). Means not designated by a common letter are different ($P < .001$).

XN + GS-induced lipolysis

Mature adipocytes were treated with either DMSO or test compounds for 12 hours, and free glycerol released was assayed. The results show that XN25 and GS50 induced lipolysis by 13.2 ± 4.6% and 21.5 ± 5.06% ($P < .05$), whereas XN25 + GS50 caused an enhanced induction of lipolysis by 44.2 ± 2.4% ($P < .001$) (Fig. 6). However, the calculated additive response would have been an increase of 34.7 ± 9.7%, which is not significantly different from the combined response, indicating an additive effect on lipolysis with the XN25 + GS50 combination (Table 1).

DISCUSSION

It is becoming evident that fat cells have a finite life span and can be eliminated by apoptosis.[1] Because adipose tissue mass can be decreased by removing adipocytes, inducing apoptosis and inhibiting adipogenesis at various stages of the adipocyte life cycle may be target pathways for treating obesity. In this study the enhanced effects of XN and GS on adipocyte adipogenesis and apoptosis were investigated.

XN and GS have each been reported to induce apoptosis and inhibit adipogenesis in 3T3-L1 adipocytes.[5,12] In the current study, the combination caused a potentiated decrease in cell viability and increase in apoptosis in mature 3T3-L1 adipocytes. XN-induced apoptosis in adipocytes was related to reactive oxygen species (ROS) generation, leading to loss of mitochondrial membrane permeability and subsequently resulting in apoptosis. Likewise, the mitochondrial pathway was reported to be involved in GS-induced apoptosis in adipocytes. Interestingly, XN + GS did not cause a potentiated increase in ROS generation (data not shown), although XN by itself increased ROS generation, which is in agreement with previous reports.[5] We then investigated the effect of XN + GS on caspase-3/7 activity and Bcl-2 family protein expression. Caspase-3 is activated during most apoptotic processes and is believed to be the main executor caspase. Consistent with previous studies,[6,18] both XN and GS increased caspase-3/7 activity, and, as expected, XN + GS further caused a potentiated increase in caspse-3/7 activity.

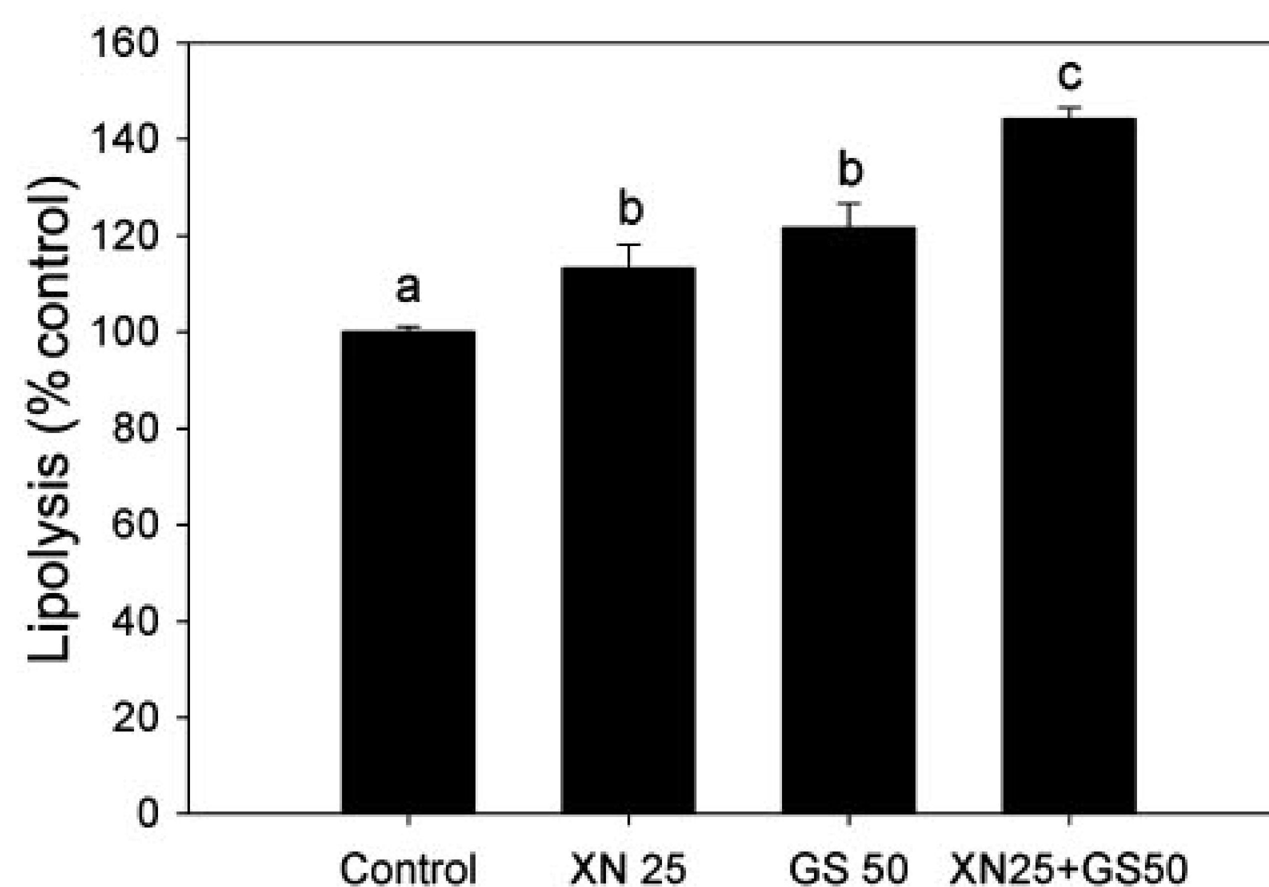


FIG. 6. Effect of XN and GS on lipolysis. Free glycerol released was assayed as described in Materials and Methods. The experiment was repeated two times with at least four replicates. Data are mean ± SEM (vertical bars) values. Means not designated by a common letter are significantly different ($P < .05$).

Bcl-2 family proteins are critical regulators of the apoptotic pathways, and alterations in the levels of Bax and Bcl-2 play an important role in determining whether cells will undergo apoptosis. A decrease in Bcl-2 expression and increase in Bax expression with XN and GS treatment was reported in cancer cells.[6,18] XN + GS-treated cells, at the concentrations tested, however, did not have altered expression levels of Bcl-2 and Bax. Interestingly, XN + GS caused an enhanced expression of Bax and reduced expression of Bcl-2 proteins, thus altering the ratio of Bax to Bcl-2 in favor of apoptosis. Therefore, we can conclude that enhanced effects of XN + GS on adipocyte apoptosis are associated with the mitochondrial pathway but without the involvement of ROS generation.

In addition to induction of apoptosis, enhanced inhibition of adipogenesis was also caused by XN + GS in maturing preadipocytes. XN and GS decreased adipocyte-specific transcription factor expression in 3T3-L1 adipocytes leading to decreased lipid accumulation.[5,12] Transcriptional factors PPARγ and C/EBPα are further involved in the sequential expression of adipocyte-specific protein aP2.[19] DGAT1, another transcription factor, catalyzes the final step in the

glycerol phosphate pathway, considered the major pathway for triacylglycerol synthesis.[20] XN was reported to decrease aP2 and DGAT1 protein expression in adipocytes at 25 μM concentration,[5] but the effect of GS on aP2 and DGAT1 was not reported. In this study, XN and GS did not alter the expression levels of adipocyte-specific proteins by themselves, but the combination caused a synergistic decrease in expression of the same. The absence of an effect of XN and GS as individual compounds on the expression of these factors can be attributed to the use of low doses of these compounds (XN1.5 and GS3.12), when compared to 25 μM used in the previous studies.

In this study, the effect of XN and GS on lipolysis was also investigated. Our finding that GS induced lipolysis in mature 3T3-L1 adipocytes is in agreement with previous reports. To our knowledge this is the first report of XN-induced lipolysis in mature adipocytes. Nevertheless, XN + GS caused an enhanced induction of lipolysis. GS was reported to activate mitogen-activated protein kinase kinase-extracellular signal-related kinase,[12] which has been shown to phosphorylate hormone-sensitive lipase and increase its lipolytic activity.[21] XN also was reported to inactivate Akt by dephosphorylation,[22] which results in the stimulation of lipolysis.[23]

The concentrations tested in the present study are less than those used in previous reports.[24,25] In an *in vivo* study, XN at 0.5 mM administered for 4 weeks did not affect any major organ functions in mice.[24] Likewise, oral administration of GS (25 mg/kg of body weight in rats)[25] was effective in decreasing plasma cholesterol levels. Although relationships between concentrations shown effective *in vitro* conditions and effective plasma levels of the same agent cannot be predicted, the combination of XN + GS would be more effective than either compound alone in decreasing adiposity *in vivo* by induction of apoptosis in mature adipocytes and by the inhibition of differentiation of preadipocytes.

CONCLUSIONS

Adipose tissue mass can be reduced by both inhibiting adipogenesis and inducing apoptosis of adipocytes. Natural products that specifically target both these pathways therefore will have better potential for treatment and prevention of obesity. XN and GS have each been shown to inhibit adipogenesis and induce apoptosis in adipocytes, but in this study we have shown that the combination of XN + GS exerts enhanced activity in both inhibiting adipogenesis and induction of apoptosis. Combinations of natural compounds have been shown to have synergistic effects in a number of biological systems, and this property has been used to advantage in many natural treatments. Our findings suggest that the combination of XN and GS may be of benefit in treating or preventing obesity.

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AUTHOR DISCLOSURE STATEMENT

C.A.B. is the CEO and Chairman of the Board of AptoTec, Inc. M.A.D.-F. is the CSO and a member of the board of directors of AptoTec, Inc. S.R., J.-Y.Y., H-J.P., and S.A. have no competing financial interests.

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