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Modes of Action of Alpha-hederin and Thymoquinone, Active Constituents of *Nigella sativa*, against HEp-2 Cancer Cells

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Abstract. *Our previous studies on active constituents of Nigella sativa have indicated that cell death induced by thymoquinone and alpha-hederin was dose- and time-dependent, in a range of four cancer cell lines. Both compounds elicited necrosis and apoptosis with a higher incidence of the latter induced by thymoquinone. As HEp-2 human laryngeal carcinoma cells were the most susceptible, we sought to better understand the mechanisms involved by using buthionine sulfoximine (BSO), a selective inhibitor of glutathione (GSH) synthesis, to determine the importance of GSH in the apoptosis elicited, using cisplatin as internal standard. BSO significantly enhanced alpha-hederin- and cisplatin- mediated toxicity as assessed by the MTT assay, without changes in apoptosis or necrosis levels. Although the MTT assay did not indicate BSO potentiation of thymoquinone, apoptosis levels were significantly enhanced following this combination, without changes in necrosis. Thymoquinone and cisplatin significantly decreased GSH levels in a dose-dependent manner, with BSO pre-treatment synergistically depleting GSH levels in only thymoquinone- treated cells. As the caspase 3 inhibitor, Z-DEVD-fmk significantly decreased thymoquinone- and cisplatin-induced apoptosis, GSH depletion and caspase 3-activation mediate thymoquinone-induced apoptosis, in this cell line.*

In a strategy combining the MTT assay of cell viability with quantification of apoptosis and necrosis, we assayed alpha-hederin and thymoquinone, constituents of *Nigella sativa*, against four cancer cell lines. Both compounds elicited necrosis and apoptosis, with a higher incidence of the latter in HEp-2 larynx carcinoma cells (IC_{50} values of 15.9 and 34.6 μM, respectively) (1). However, the mechanisms of action were not established. Intracellular glutathione (GSH), a tripeptide thiol found in virtually all cells (2), functions in cellular protection, synthesis and repair of DNA, and its extrusion is associated with apoptosis (3-6); it significantly affects the toxicity of anti-cancer drugs (7). Pre-treatment with buthionine sulfoximide (BSO), highly active as an inhibitor of γ-glutamylcysteine synthetase in GSH biosynthesis (8), may render cells more sensitive to the induction of apoptosis (9, 10). BSO pre-treatment augments alpha-hederin-induced apoptosis in a leukaemia cell line (11), and here we explore a corresponding effect of that compound on the relatively low levels of apoptosis induced in the HEp-2 cell line in comparison with that induced by thymoquinone (1), using cisplatin as an internal standard. Thymoquinones mechanisms of apoptosis involve: up-regulation of Bax and a decrease in Bcl-2 in primary mouse keratinocytes papilloma (SP-1 cells) (12); p-53 dependency and decreased Bcl-2 levels in human colorectal (HCT116) cancer cells (13); and caspase dependency in human leukaemia (HL-60) cells (14). We now report on the role of GSH in apoptosis induced by thymoquinone, which has not been previously examined.

Accordingly, the aims of this study were to examine: firstly, potentiation of alpha-hederin and thymoquinone by BSO, by use of the MTT assay and apoptosis/necrosis quantification; and secondly, if apoptosis is induced *via* GSH depletion, and caspase-3 activation, using cisplatin throughout for comparison.

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Materials and Methods

Drugs. Alpha-hederin and thymoquinone stock solutions were made up in 100% DMSO and cisplatin and BSO in dH_2O. All chemicals were purchased from Sigma-Aldrich Ireland Ltd; unless otherwise stated.

Human cell culture. HEp-2 (larynx carcinoma) cells were obtained from the National Cell and Tissue Culture Centre, Dublin City University, Dublin, Ireland, and were maintained as previously described (15).

Cytotoxicity assay. To assess the effect of BSO as a potentiator of toxicity, cells in 96-well plates were incubated with BSO (10 or 100 μM) for 24 h followed by treatment with alpha-hederin (8-20 μM), thymoquinone (25-100 μM) or cisplatin (30 μM) in 8 replicates for a further 24 h. Cellular viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (1).

Apoptosis and necrosis quantification. Cells grown on coverslips in 35-mm tissue culture dishes were treated with BSO (10 μM) for 24 h, followed by either alpha-hederin (14 μM), thymoquinone (50 μM) or cisplatin (30 μM) for a further 24 h. Apoptosis and necrosis quantification was as previously described (1). For caspase 3-inhibition, cells were treated with 100 μM Z-DEVD-fmk (R&D systems, UK) for 2 h prior to treatment with thymoquinone (50 μM) or cisplatin (30 μM). Each experiment was carried out in duplicate and repeated at least twice.

Glutathione quantification. Cells treated with BSO (10-500 μM), thymoquinone (25-100 μM), and cisplatin (30-100 μM) were assayed for GSH content. In addition, cells pre-treated with BSO (10, 100 μM), then with thymoquinone (25, 50 μM) or cisplatin (30 μM) were also quantified. Total glutathione was carried out as previously described (16), using a slight variation of the Tietze assay (17) as modified by Cribb (18). Total protein concentration was determined with a Pierce BCA protein kit (19), such that the GSH concentration could be expressed as nmol/mg of total protein. Each experiment was carried out in triplicate and repeated twice.

Drug synergism. The CI (combination index) was calculated to determine whether BSO acted synergistically with thymoquinone or cisplatin in the depletion of GSH, where $CI=(D)_1/(Dx)_1+(D)_2/(Dx)_2$. $(D)_1$ and $(D)_2$=the dose of drug 1 and 2 that elicits a certain effect in the combination; $(Dx)_1$ and $(Dx)_2$=the dose of drug 1 and 2 that elicits an identical effect by itself. CI>1 indicates antagonism, CI=1 indicates additivity, and CI<1 indicates synergy (20).

Statistical analysis. Data are presented as mean±S.E.M and analysed by ANOVA using SPSS, followed by Bonferroni *post hoc* analysis for multiple comparisons, also for comparing observed *vs.* expected effects in combination experiments.

Results

BSO has variable effects on cytotoxicity induced by alpha-hederin, thymoquinone and cisplatin. Treating HEp-2 cells with BSO (10, 100 μM) prior to adding alpha-hederin, thymoquinone or cisplatin had varying effects on cellular viability as assessed by the MTT assay (Figure 1A,B). Pre-treatment with 10 μM BSO did not enhance the effect of 8 or 12 μM alpha-hederin, but 100 μM BSO significantly ($p<0.05$) enhanced (1.3-fold) cytotoxicity of 12 μM alpha-hederin compared with alpha-hederin alone, decreasing cellular viability from 46.3±3.6% to 35.8±0.3% (Figure 1A). Neither concentration of BSO tested was singly cytotoxic to HEp-2 cells. Pre-treatment with 10 and 100 μM BSO followed by 16 μM alpha-hederin significantly ($p<0.05$ and <0.01 respectively) enhanced (1.4-fold) cytotoxicity, decreasing cellular viability to 29.6±2.2 and 28.7±0.5%, respectively, from 40.8±2% attributable to 16 μM alpha-hederin alone (Figure 1A). Treatment with BSO (10, 100 μM) did not significantly enhance the efficacy of thymoquinone at any concentration tested (data not shown). Although 10 μM BSO did not increase cisplatin cytotoxicity, 100 μM pre-treatment significantly ($p<0.001$) enhanced (2.1-fold) the effect of 30 μM cisplatin, decreasing viability from 50±3.6% to 23.8±2% (Figure 1B).

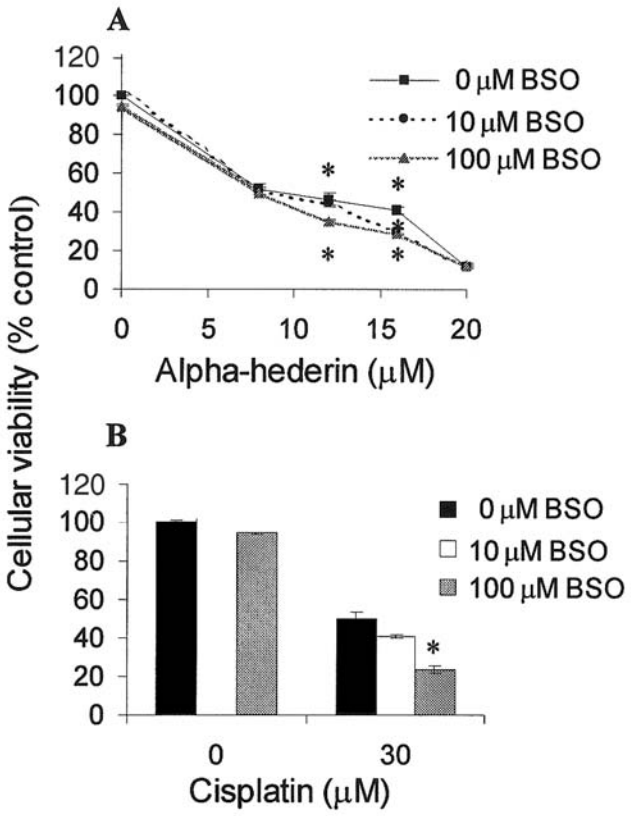

Figure 1. *Effect of BSO (10, 100 μM) pre-treatment (24 h) followed by alpha-hederin (A) (8-20 μM) (24 h) and cisplatin (B) (30 μM) on HEp-2 cellular viability as assessed by the MTT assay. Results are expressed as % of the controls and represent 2 independent experiments (±S.E.M). * Indicates a statistically significant ($p<0.05$) difference between single and combined treatments.*

BSO enhances apoptosis induced by thymoquinone but not by alpha-hederin, or cisplatin. To further assess the use of BSO as a possible enhancer, through GSH inhibition, of the effects of alpha-hederin, thymoquinone and cisplatin, the extent of apoptosis and necrosis was quantified.

BSO alone did not change apoptosis/necrosis levels from control levels. Pre-treatment with 10 μM BSO followed by thymoquinone (50 μM) significantly ($p<0.01$) increased apoptosis 1.7-fold (31.7±6.1% to 55.1±7.3%), with no significant change in necrosis (Figure 2). Pre-treatment followed separately by alpha-hederin (14 μM) and cisplatin (30 μM) did not significantly enhance apoptosis or necrosis (Figure 2).

Thymoquinone and cisplatin deplete GSH levels. Treating HEp-2 cells with increasing concentrations of BSO for 24 h depleted GSH content dose-responsively (data not shown).

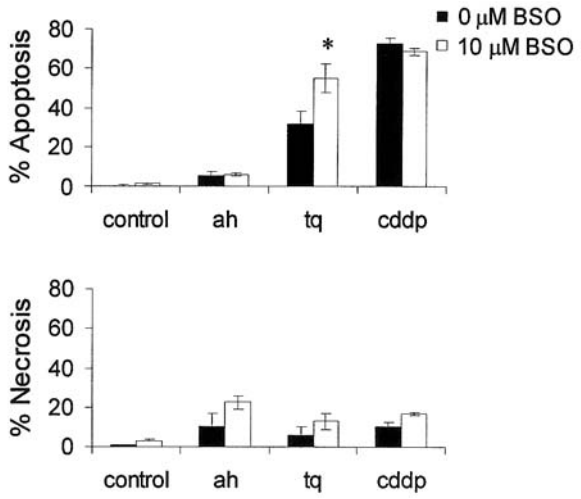

Figure 2. *The effect of 10 μM BSO pre-treatment (24 h) followed by Tq (50 μM thymoquinone), Ah (14 μM alpha-hederin) or Cddp (30 μM cisplatin) (24 h) on apoptosis and necrosis of HEp-2 cells. Apoptosis and necrosis were quantified using Hoechst and trypan blue staining methods, respectively. Results are expressed as % of cell population and represent at least 2 independent experiments (±S.E.M). * Indicates a statistically significant difference ($p<0.01$) from single treatments.*

Treating HEp-2 cells with increasing concentrations of thymoquinone decreased GSH levels dose-responsively as: 25 and 50 μM thymoquinone had a negligible effect (<8% depletion); 75 μM depleted GSH levels by 28.1±11.2%; and 100 μM significantly ($p<0.01$) depleted GSH levels by 71±10.7% (Figure 3A). Increasing concentrations of cisplatin also dose-responsively depleted GSH levels as: 30 μM depleted by 26.5±10.9%; and 50 and 100 μM cisplatin significantly ($p<0.01$ and 0.001, respectively) depleted by 88.2±10.5% and 94.8±0.5%, respectively (Figure 3B).

Effects of BSO pre-treatment on GSH depletion by thymoquinone and cisplatin. BSO (10, 100 μM) followed by 25 μM thymoquinone did not significantly deplete GSH (52.1±7.9%, 68.6±8.6%, respectively) compared with 10 and 100 μM BSO alone (42.5±10.9, 59.8±1.1%, respectively), and thymoquinone alone (0±1%). However, 10 μM BSO followed by 50 μM thymoquinone had a significant ($p<0.05$) effect: 94.8±3.2% depletion, compared with 42.5±10.9% (10 μM BSO alone), and 8.9±11.9% (50 μM thymoquinone alone). BSO (10, 100 μM) followed by 30 μM cisplatin did not significantly deplete GSH levels (64.3±9.8 and 88.3±5.2%, respectively) compared with BSO alone (Figure 4).

To explore possible synergies, GSH depletion induced by thymoquinone alone, cisplatin alone, BSO alone and BSO in combination with each, were plotted to calculate combination indexes (CI), *i.e.* the concentration of each drug singly that produces an effect equal to the combination (plots not shown). Thymoquinone and BSO (10 μM) acted synergistically to deplete HEp-2 GSH compared with single treatments (CI=0.39±0.06 $p<0.05$), whereas with higher concentrations of BSO (100 μM) in the combination, the effect is simply additive (CI=1.01±0.01) (Table I). CI values for cisplatin and BSO (10, 100 μM) of 0.84±0.25 and 1.1±0.05, respectively, also were additive (CI approaching and equal to 1, respectively).

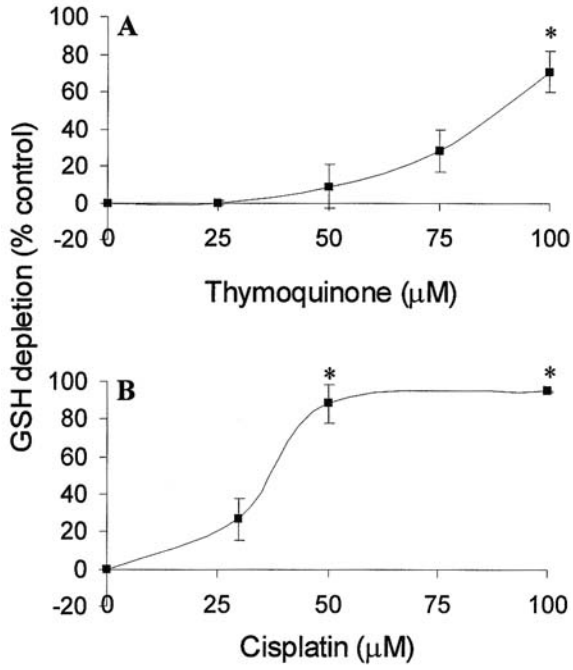

Figure 3. *Effects of increasing concentrations of thymoquinone (A) and cisplatin (B) on HEp-2 GSH levels, determined by the modified method of Popat (16) and Cribb (18). Results are expressed as % control of GSH nmoles/mg, and represent at least 2 independent experiments, each performed in triplicate (±S.E.M). * Indicates a statistically significant difference between control and treatments ($p<0.01$).*

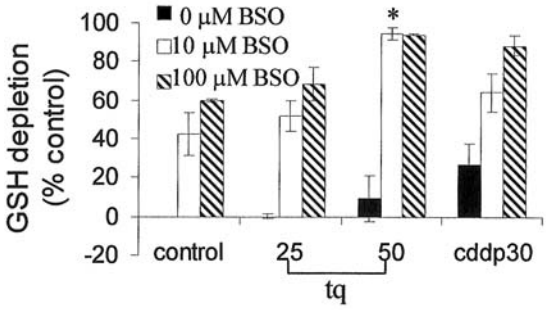

Figure 4. *Effects of BSO pre-treatment (10, 100 μM) (24 h) followed by Tq (25, 50 μM thymoquinone) or Cddp (30 μM cisplatin) on HEp-2 cell GSH levels, determined by the modified method of Popat (16) and Cribb (18). Results are expressed as % control of GSH nmoles/mg, and represent at least 2 independent experiments, each performed in triplicate (±S.E.M). * Indicates statistically significant difference between combined and single treatments ($p<0.01$).*

Table I. *Effects of BSO, thymoquinone (Tq), and cisplatin (Cddp) singly, and in combination, on HEp-2 cell GSH depletion. The concentrations used are the GD_{70} (μM) (doses yielding 70% GSH depletion), compared with the combined doses eliciting the same effect.*

GD_{70} (μM)	
BSO 122±3.5 Tq 101±7.8 Cddp 48.8±0.8	
Combination Index	
BSO(10 μM)+TQ(31 μM) 0.39±0.06*	BSO(100 μM)+TQ(19 μM) 1.01±0.01
BSO(10 μM)+Cddp(37 μM) 0.84±0.25	BSO(100 μM)+Cddp(11 μM) 1.1±0.05

*Indicates synergy

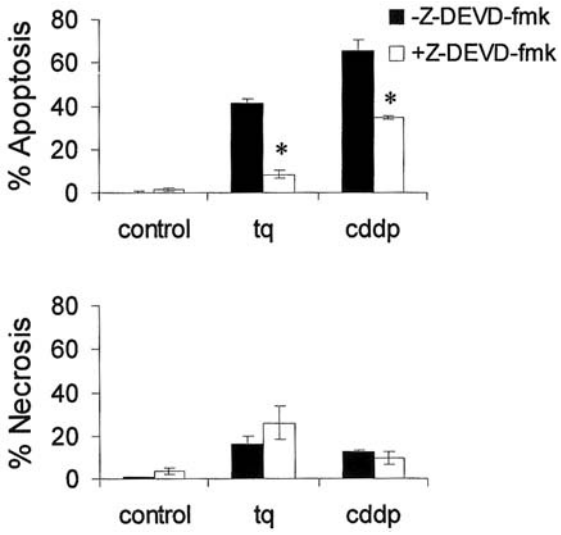

Figure 5. *The effect of Tq (50 μM thymoquinone), and Cddp (30 μM cisplatin) on apoptosis and necrosis of HEp-2 cells in the absence (–) or presence (+) of caspase 3-inhibitor (Z-DEVD-fmk, 100mM). Apoptosis and necrosis were quantified using Hoechst and trypan blue staining methods, respectively. Results are expressed as % of cell population and represent 2 independent experiments (±S.E.M). * Indicates a statistically significant difference ($p<0.01$) from single treatments.*

Caspase 3-inhibitor decreases apoptosis but not necrosis induced by thymoquinone and cisplatin. The caspase 3 inhibitor (Z-DEVD-fmk, 100 μM) significantly decreased (4.9-fold) thymoquinone-induced apoptosis compared with thymoquinone alone ($p<0.01$); (41.1±2.1% to 8.4±1.7%), with no significant effect on necrosis (15.9±3.5% compared with 25.9±7.7%) (Figure 5). This inhibitor also significantly decreased cisplatin-induced apoptosis (1.9-fold) from 65.6±4.8% to 34.5±0.8% ($p<0.01$), with no significant effect on necrosis (12.2±1% compared with 9.5±3% necrosis).

Discussion

In this study of the *in vitro* modes of action of alpha-hederin and thymoquinone, in HEp-2 carcinoma cells, BSO significantly enhanced thymoquinone-induced apoptosis, but not that induced by either alpha-hederin or cisplatin. The lack of BSO-enhanced apoptosis taken with the low quantities of apoptosis induced by alpha-hederin alone obviated the need for further investigation of this compound with this cell line.

Both thymoquinone- and cisplatin-induced apoptosis occurred *via* GSH depletion, the effect of which was synergistically enhanced by BSO in thymoquinone-treated cells. This has not been previously shown.

Although the MTT assay detected no significant change in thymoquinone-induced cytotoxicity following pre-treatment with BSO, the combination significantly increased apoptosis levels. Enhanced cytotoxicity does not always co-occur with enhanced apoptosis/necrosis. Tirapazamine was synergistically cytotoxic with BSO against human neuroblastoma cell lines, without a corresponding increase in apoptosis (21). In contrast, pre-treatment with BSO, enhanced apoptosis in: liver cholongiocytes treated with beauvericin (22); human colorectal cancer cells treated with melphalan (23); and in human small cell lung carcinoma (24). This variable effect may be cell line dependent, as high intracellular GSH levels correlate with resistance (25).

With respect to the mechanism of apoptosis, our data show that only high concentrations of thymoquinone (>75 μM) significantly depleted GSH levels, despite maximum apoptosis induced by 50 μM. Similarly, cells treated with cisplatin displayed significant GSH depletion only with higher concentrations of 50 and 100 μM, although high levels of apoptosis were induced with 30 μM. Apparently, GSH depletion is associated with a very early event in the processes eliciting apoptosis but is not essential, in this and other cell lines. Similarly, treating colon adenocarcinoma cells with capillin induced significant apoptosis with no significant change in GSH levels (15). Previous studies have also demonstrated cytotoxicity in response to cisplatin without GSH depletion in a human melanoma cell line (25).

GSH depletion *via* BSO, prior to the addition of thymoquinone, synergistically depletes GSH, as 10 μM BSO with 50 μM thymoquinone deplete GSH levels by 95%, and coupled with the enhanced apoptosis levels observed, might have therapeutic significance. BSO interaction with other natural compounds is variable; potentiating leukaemia cell apoptosis by alpha-hederin (11), but antagonistic to the effect of taxol on MCF-7 breast adenocarcinoma and A549 lung adenocarinoma cells (26). The failure of pre-treatment with BSO to enhance GSH depletion by cisplatin has not been previously reported in this cell line, and the lack of apoptotic

enhancement concurs with studies on B.5 LX-1 human small cell carcinoma (24) and KB human oropharyngeal epidermoid carcinoma cells (27).

Our confirmation of the involvement of caspase 3, is consistent with the pathway of thymoquinone-induced apoptosis in neoplastic keratinocytes (12), and that of caspase 8, 9 and 3 activation in a human leukaemia cell line (14). The 50% decrease in cisplatin apoptosis indicates the involvement of both caspase 3-activation and a caspase-3 independent pathway.

The present data demonstrate, for the first time, the role of GSH depletion and caspase 3-involvment in thymoquinone-induced apoptosis in a laryngeal cancer cell line. The synergistic enhancement by BSO calls for further studies using this inhibitor in conjunction with thymoquinone.

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