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Cytotoxic Activity of Benzo[*b*]cyclohept[*e*][1,4]oxazines

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Abstract. *Background: Although numerous articles have dealt with the biological activities of azulenes, studies of benzo[b]cyclohept[e][1,4]oxazines are limited. In the present study, we investigated a total of 14 newly-synthesized benzo[b]cyclohept[e][1,4]oxazines for their growth stimulation at low concentrations (so-called 'hormesis'), cytotoxicity at higher concentrations and apoptosis-inducing activity. Materials and Methods: Cytotoxicity of these compounds against human normal gingival fibroblast (HGF) and human oral squamous cell carcinoma cell lines derived from gingival tissue (Ca9-22), was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The tumor specificity (TS) was determined by the ratio of the 50% cytotoxic concentration (CC_{50}) value for HGF cells to that for Ca9-22 cells. Apoptosis induction was evaluated by DNA fragmentation and caspase-3 activation. Results: Compounds 10-(2-methoxyethylamino)benzo[b] cyclohept[e][1,4]oxazine and 10-(3-methoxypropylamino) benzo[b]cyclohept[e][1,4] oxazine, but not other compounds, induced hormesis only in HGF cells. Compound 10-(6-hydroxyhexylamino)benzo[b] cyclohept[e][1,4]oxazine [**4**] showed the highest cytotoxicity against Ca9-22 cells, followed by 10-(4-hydroxybutylamino) benzo[b]cyclohept[e] [1,4]oxazine and 10-(5-hydroxypentyl-amino)benzo[b]cyclo-hept[e][1,4]oxazine. Compound [**4**] did not induce apoptosis markers, but rather induced necrotic cell death (characterized by a smear pattern of DNA fragmentation). Conclusion: The present study suggests that the OH group and a certain length of methylene group are necessary for maximal cytotoxicity, and substitution of fluoride in the benzene ring enhances cytotoxicity.*

Azulene, an isomer of naphthalene, has a dipole moment and a resonance energy with intermediate values between that of benzene and naphthalene, and is considerably more reactive when compared with two arenes. The synthesis and chemical reactions of azulene derivatives have been investigated (1-3). They have been reported to have anti-bacterial (4), anti-ulcer (5), and relaxant activities (6), to inhibit thromboxane A_2-induced vasoconstriction and thrombosis (7), and have been investigated for acute toxicity and local anesthetic activity (8), and chemotherapeutic activity against mucous membrane diseases (9, 10). We have reported their tumor specificity (11-16), inhibitory action against nitric oxide production by activated macrophages (17-23), and anti-UV activity (24-26). However, studies of benzo[*b*]cyclohept[*e*][1,4]oxazines are limited. Therefore, in the present study, we investigated a total of 14 newly-synthesized benzo[*b*]cyclohept[*e*][1,4]oxazines [**1-14**] (Figure 1) for their cytotoxicity against human normal oral gingival fibroblast (HGF) and human oral squamous cell carcinoma cells derived from gingival tissues (Ca9-22).

It has been reported that many toxic substances, environmental hormones, inorganic compounds, and even irradiation induce hormesis that modulate the growth of cultured cells in a biphasic fashion, stimulating or inhibiting the growth of cultured cells at low and high concentrations, respectively (27, 28). However, we recently found that azulenes (25, 26), tropolones (24), 2-aminotropones (29), sodium fluoride (30), Chinese herbal extracts (31), and a low level of CO_2 laser irradiation (32, 33) induced hormesis in oral cells to a limited extent, or only for certain durations and concentrations. To confirm the generality of the occurrence of hormesis, we investigated whether benzo[*b*]cyclohept[*e*][1,4]oxazines induce hormesis in HGF and Ca9-22 cells.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) from GIBCO BRL, Grand Island, NY, USA; fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich Inc., St. Louis, MO, USA; dimethyl sulfoxide (DMSO) from Wako Pure Chem. Ind., Osaka, Japan; and caspase-3 substrate [DEVD-*p*NA (*p*-nitroanilide)] from MBVL, Nagoya, Japan.

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Key Words: Benzocycloheptoxazines, hormesis, cytotoxicity.

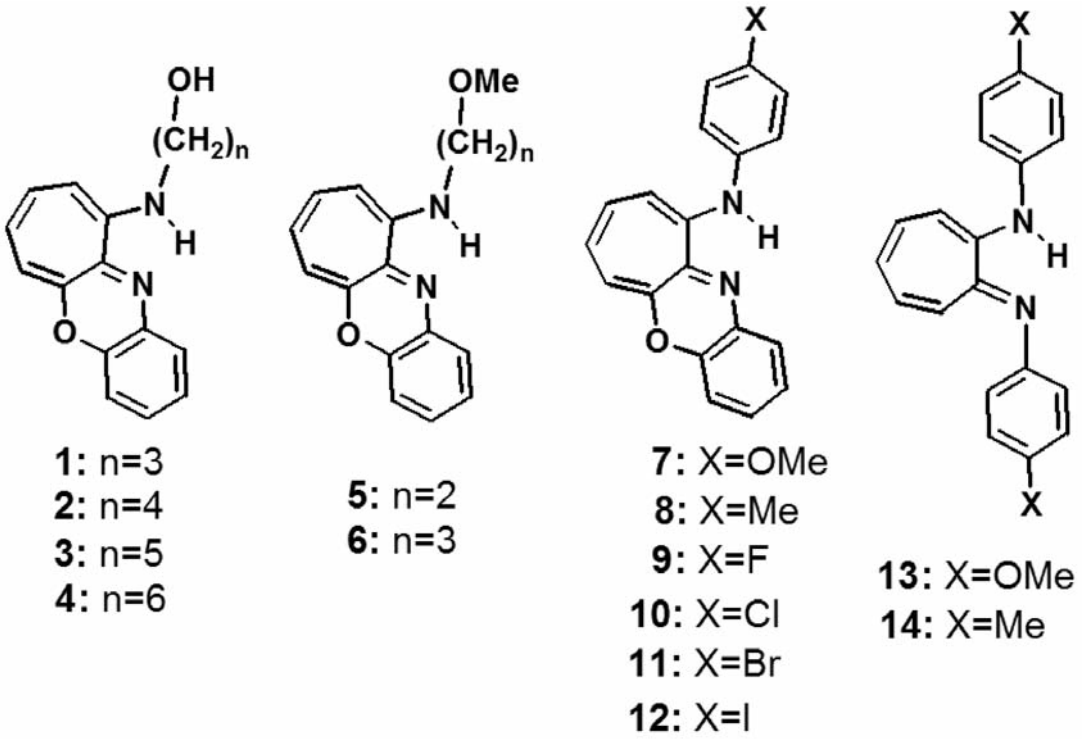

Figure 1. *Chemical structure of fourteen benzo[b]cyclohepta[e][1,4]oxazines: 10-(3-hydroxypropylamino)benzo[b]cyclohept[e][1,4]oxazine [**1**], 10-(4-hydroxybutylamino)benzo[b]cyclohept[e][1,4]oxazine [**2**], 10-(5-hydroxypentylamino)benzo[b]cyclohept[e][1,4]oxazine [**3**], 10-(6-hydroxyhexylamino)benzo[b]cyclohept[e][1,4]oxazine [**4**], 10-(2-methoxyethylamino)benzo[b]cyclohept[e][1,4]oxazine [**5**], 10-(3-methoxypropylamino) benzo[b]cyclohept[e][1,4]oxazine [**6**], 10-(4-methoxyanilino)benzo[b]cyclohept[e][1,4]oxazine [**7**], 10-(4-methylanilino)benzo[b]cyclohept[e] [1,4]oxazine [**8**], 10-(4-fluoroanilino)benzo[b]cyclohept[e][1,4]oxazine [**9**], 10-(4-chloroanilino)benzo[b]cyclohept[e][1,4]oxazine [**10**], 10-(4-bromoanilino)benzo[b]cyclohept[e][1,4]oxazine [**11**], 10-(4-iodoanilino)benzo[b]cyclohept[e][1,4]oxazine [**12**], N,N'-bis(4-methoxypheny1) aminotroponimine [**13**] and N,N'-bis(4-methylpheny1)aminotroponimine [**14**].*

Synthesis of test compounds.
10-(3-Hydroxypropylamino)benzo*[b]*cyclohept[*e*][1,4]oxazine [**1**], 10-(4-hydroxybutylamino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**2**], 10-(5-hydroxypentylamino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**3**], 10-(6-hydroxyhexylamino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**4**], 10-(2-methoxyethylamino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**5**], 10-(3-methoxypropylamino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**6**], 10-(4-methoxyanilino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**7**], 10-(4-methylanilino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**8**], 10-(4-fluoroanilino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**9**], 10-(4-chloroanilino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**10**], 10-(4-bromoanilino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**11**], 10-(4-iodoanilino)benzo[*b*]cyclohept[*e*][1,4]oxazine [**12**], *N,N'-bis*(4-methoxypheny1)aminotroponimine [**13**] and *N,N'-bis*(4-methylpheny1)aminotroponimine [**14**] (Figure 1) were synthesized according to previous references (15, 24, 34, 35). All compounds were dissolved in DMSO at 50 mM. The final DMSO concentration subsequently added to the cells was less than 0.5% that did not affect the normal cellular growth.

Cell culture. HGF cells were established from a first premolar tooth extracted from the lower jaw of a 12-year-old girl, as described previously (24). Ca9-22 cells were provided by Riken Cell Bank (Tsukuba, Japan).

Assay for cytotoxic activity. Cells were inoculated at 3×10^3 cells/0.1 ml in the inner 60 wells of a 96-microwell plate (Becton Dickinson Labware, NJ, USA). The surrounding 36 exterior wells were filled with 0.1 ml of phosphate-buffered saline without calcium and magnesium [PBS(–)] to minimize the evaporation of water from the culture medium. After 48 h, the medium was removed by suction with an aspirator, and replaced with 0.1 ml of fresh medium containing different concentrations of single test compounds (1.9-500 μM). Cells were incubated for 48 h, and the relative viable cell number was then determined by the MTT method (25, 26). In brief, the treated cells were incubated for another 3 h in fresh culture medium containing 0.2 mg/ml MTT. Cells were then lysed with 0.1 ml of DMSO, and the absorbance at 540 nm of the cell lysate was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve and the mean value of CC_{50} for each cell type was calculated from 3 independent experiments. The tumor-specificity index (TS) was determined by the following equation:

$TS=CC_{50}[HGF]/CC_{50}[Ca9\text{-}22]$.

Assay for hormesis. The hormetic response was evaluated by the maximum response in each dose-response curve, as described previously (24, 25, 26, 29).

Table I. *Hormetic response of human oral normal and tumor cells after treatment with benzocycloheptoxazines.*

Compd.	Maximum hormetic response (%)	
	HGF	Ca9-22
1	14.3±6.1	9.6±8.6
2	18.7±5.9	7.07±12.2
3	18.32±9.1	21.83±9.6
4	26.4±8.1	3.56±6.2
5	137.47±19.9	25±20.6
6	140.29±11.3	1.87±3.2
7	34.34±6.3	11.71±14.9
8	24.82±6.8	14.55±18.3
9	3.77±3.0	11.98±11.2
10	15.04±3.3	23.37±26.0
11	27.8±14.2	22.95±18.6
12	10.3±9.2	15.49±13.4
13	17.57±3.5	2.52±4.4
14	32±16.7	11.19±12.2

HGF: Human gingival fibroblast. Each value represents experimental data from three independent experiments which were carried out in triplicate.

Table II. *Cytotoxicity of benzocycloheptoxazines towards human oral normal and tumor cells.*

Compd.	CC_{50} (μM)		
	HGF	Ca9-22	TS
1	484.6±26.7	347.4±178.2	>1.39
2	154.2±35.8	98.8±9.8	1.56
3	140.3±47.5	98.9±18.3	1.42
4	134.2±31.0	62.8±11.7	2.14
5	>500	>371.5±222.6	><1.35
6	>500	>372.0±221.6	><1.34
7	>500	>500	><1.00
8	>500	383.8±153.2	>1.30
9	>500	212.8±52.6	>2.35
10	>500	>485.3±14.8	><1.03
11	>500	>500	><1.00
12	>500	381.8±78.8	>1.31
13	>500	172.3±59.9	>2.90
14	>500	487.4±14.3	><1.03

HGF: Human gingival fibroblast; TS: tumor specificity, defined as the ratio of CC_{50}[HGF]/CC_{50}[Ca9-22]. Each value represents experimental data from three independent experiments which were carried out in triplicate.

Assay for DNA fragmentation. The cells (4×10^4) were seeded on a 6-microwell plate and incubated for 48 h to allow complete attachment. The medium was removed by suction with an aspirator, and replaced with 2 ml of fresh medium containing different concentrations of single test compounds. Cells were then incubated for a further 6 or 24 h. After washing twice with PBS(–), cells were collected by scraping with a rubber policeman on ice and spun down in an eppendorf tube. Cells were lysed with 50 μl lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate]. The solution was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 2 h at 50˚C and then mixed with 50 μl NaI solution [40 mM Tris-HCl (pH 8.0), 7.6 mM NaI, 20 mM EDTA-2Na] followed by 250 μl of ethanol. After centrifugation for 20 minutes at 20,000 ×g, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA-2Na]. Each sample (10-20 μl, equivalent to 2×10^5 cells) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA-2Na). After staining with ethidium bromide, the DNA was visualized by UV irradiation, and photographed as described previously (25, 26). DNA from apoptotic HL-60 cells induced by UV irradiation (6 J/m^2/min, 1 min) (36) was run in parallel as positive controls.

Assay for caspase-3 activation. Cells (8×10^4) were seeded on a 6-microwell plate, and incubated for 48 h to allow complete adherence. Cells were incubated for 6 or 24 h with different cocentrations of test samples in fresh culture medium. Cells were washed twice with PBS(–) and lysed with 100 μl of lysis solution [50 mM Tris-HCl, pH 7.5, 0.3% NP-40, 1 mM dithiothreitol (DTT)]. Cells were collected by scraping with a rubber policeman into an eppendorf tube. After standing the tube for 10 min on ice and centrifugation for 5 min at 10,000×*g*, the supernatant was collected. Lysate (50 μl, equivalent to 100 μg protein) was mixed with 50 μl lysis solution containing a substrate for caspase-3 substrate (DEVD-*p*NA). After incubation for 24 h at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by microplate reader as described previously (25, 26).

Statistical analysis. The difference between two groups was evaluated by Student's *t*-test.

Results

Induction of hormesis. Compounds [**5**] and [**6**] significantly ($p<0.05$) induced apparent hormetic stimulation of growth of HGF cells (maximum hormetic response=137 and 140%, respectively), but not that of Ca9-22 cells (maximum hormetic response=25% or less) (Table I). On the other hand, none of the other twelve compounds induced apparent hormesis (maximum hormetic response=34% or less) (Table I).

Tumor specificity. Compound [**4**] showed the highest cytotoxicity against Ca9-22 cells, followed by [**2**] and [**3**]. The other eleven compounds [**1**, **5-14**] showed much lower cytotoxicity (CC_{50}>170 μM) (Table II). Although compounds [**13**] and [**9**] had the highest TS (>2.35), their cytotoxicity against Ca9-22 cells was very weak and they were, therefore, not subjected to further analysis. Compound [**4**] had the highest TS value among the remaining 12 compounds, followed by compound [**2**]. Compounds [**2**] and [**4**] were subjected to further analysis.

Type of induced cell death. Compounds [**2**] and [**4**] did not induce inter-nucleosomal DNA fragmentation (such as that observed in UV-induced apoptotic HL-60 cells) in other HGF

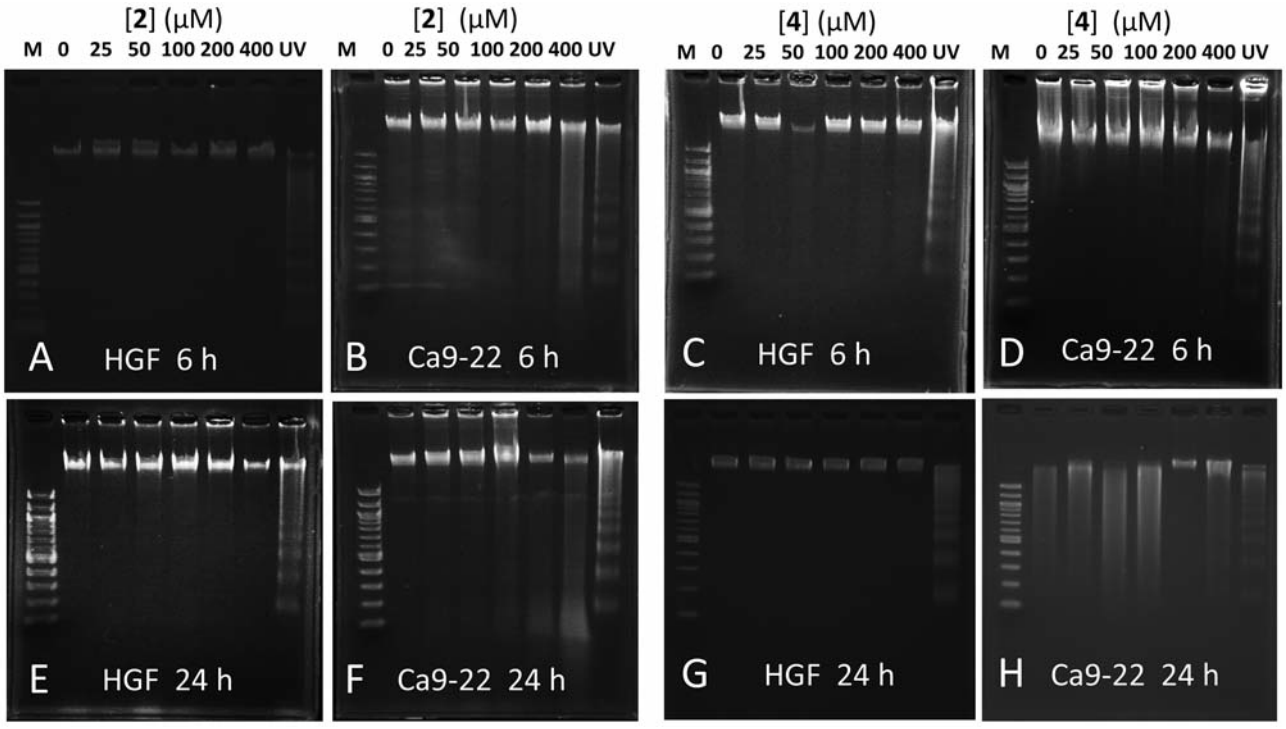

Figure 2. *Effect of benzo[b]cyclohepta[e][1,4]xazines on induction of DNA fragmentation. Human gingival fibroblast (HGF) (A, C, E, G) and Ca9-22 cells (B, D, F, H) were incubated for 6 (A-D) or 24 h (E-H) with the indicated concentrations of [**2**] (A, B, E, F) or [**4**] (C, D, G, H), and lysed for the assay of DNA fragmentation by agarose gel electrophoresis. UV, DNA from UV-irradiated HL-60 cells.*

or Ca9-22 cells (Figure 2). Compound [**4**] (50, 100 μM) rather induced a smear pattern of DNA fragmentation (Figure 2H) in Ca9-22 cells. Compounds [**2**] and [**4**] did not significantly increase caspase-3 activity in HGF cells, in contrast to the significantly ($p<0.05$) higher level of capase-3 activity in the positive control (UV-irradiated HL-60 cells) (Figure 3).

Discussion

The present study demonstrated that compounds [**5**] and [**6**] significantly induced hormetic growth stimulation of HGF cells. Since these compounds had very weak cytotoxicity against both HGF (CC_{50}>500 μM) and Ca9-22 cells (CC_{50}>372 μM), and low TS (TS=1.3), no clear-cut relationship was found between the intensity of hormesis and that of cytotoxicity or TS. Further study is required to elucidate the biological significance of this finding.

The present study demonstrated that compounds [**2-4**] had higher cytotoxicity than other compounds, suggesting that the presence of an OH group at C-3 and a certain length of the methylene group (n=4-6) are necessary for cytotoxicity induction. Compound [**7**], lacking a methylene group, exerted a lower cytotoxicity (CC_{50}>500 μM), as compared with compounds [**5**] and [**6**] with two or three methylene groups. Among 10-(4-haloanilino)benzo[*b*]cyclohept[*e*][1,4]oxazines [**9-12**], compound [**9**] with fluoride in the benzene ring had the highest cytotoxicity and a higher TS value as compared with compounds substituted with other halogens [**10-12**]. This finding is consistent with our previous report that fluorinated compounds such as trifluoroacetylazulenes (13) and hexafluorotrihydroxyvitamin D_3 derivatives exerted a higher cytotoxic or monocytic differentiation-inducing activity (37). Compounds [**13**] and [**14**] have a different backbone structure, and therefore structure/activity comparison with other groups is not possible.

The present study also demonstrated that [**4**] had the highest cytotoxicity and TS. This compound did not induce inter-nucleosomal DNA fragmentation nor capase-3 activity. Considering that this compound did induce a DNA smear pattern, necrotic cell death induction is suggested.

In conclusion, [**4**] showed some tumor-specificity towards Ca9-22 tumor cells, and [**5**] and [**6**] induced a hormetic response in HGF cells. These biological activities did not overlap. It remains to be investigated whether [**4**] enhances the antitumor activity of chemotherapeutic agents, and whether [**5**] and [**6**] aggravate gingival hyperthrophy induced as side-effects of anticancer drugs (38, 39).

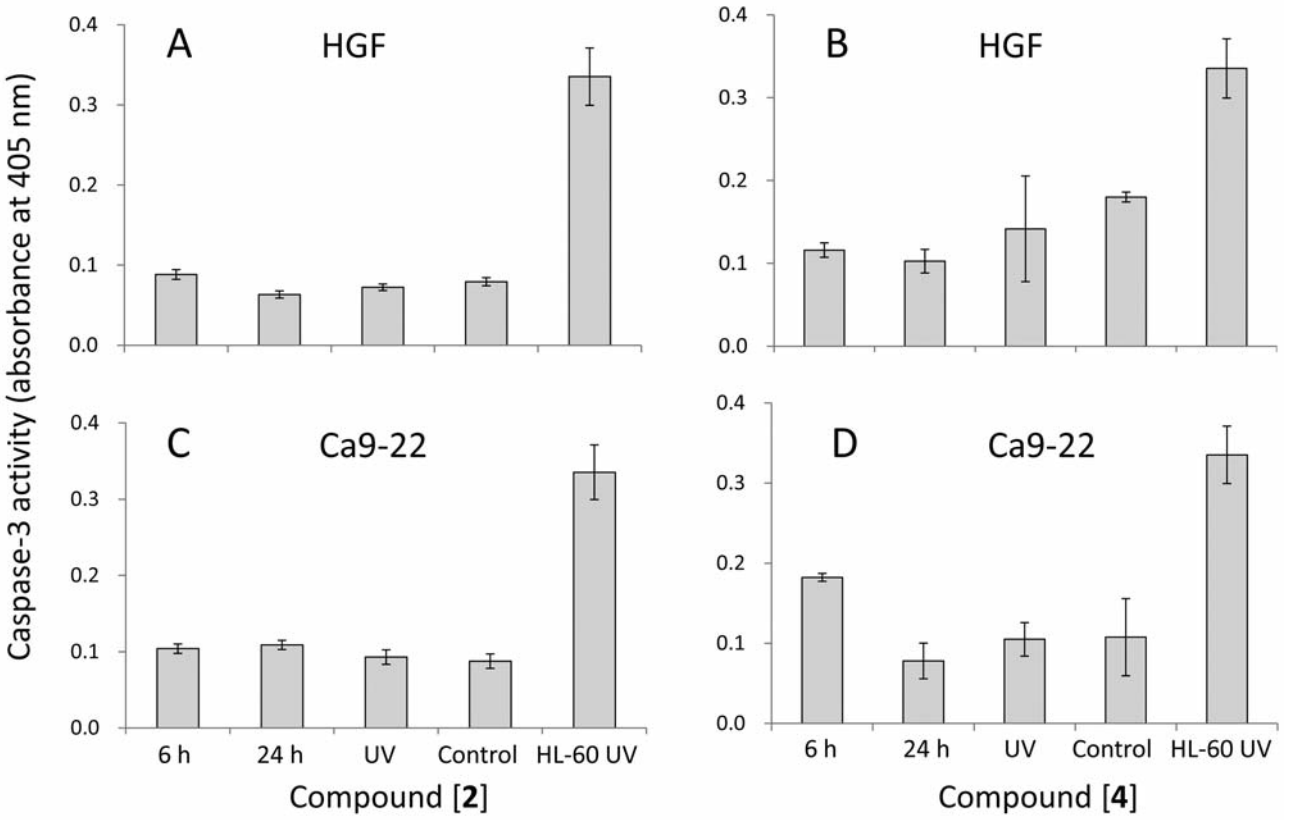

Figure 3. *Effect of benzo[b]cyclohepta[e][1,4]xazines on induction of caspase-3 activity. Human gingival fibroblast (HGF) (A, B) and Ca9-22 cells (C, D) were incubated for 6 or 24 h without (control) or with 50 μM [**2**] (A, C) or [**4**] (B, D). The cells were lysed for determination of caspase-3 activity. UV: HL-60 cells induced to apoptosis by UV irradiation were used as positive control. Caspase-3 activity was expressed as the absorbance at 405 nm. Each value represents the mean±S.D. of triplicate assays.*

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