IONIC DISSOLUTION PROFILES OF COMBINED GONIOTHALAMIN AND BIOACTIVE GLASS 45S5 IN CULTURE MEDIUM OF HUMAN OSTEOSARCOMA SAOS-2 CELLS

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Abstract

This study aimed to investigate the combined effects of goniothalamin and bioactive glass (GTN-BG) on human osteosarcoma Saos-2 cells that emphasise the extracellular changes in the cells' growth medium, including pH and ion release profile. The MTT assay was used to examine the effect of a combination dose of GTN-BG on Saos-2 cells in comparison to untreated and treated Saos-2 with bioactive glass (BG), goniothalamin (GTN), and doxorubicin (DOX). The pH of the culture media for Saos-2 cells was recorded using a pH meter. The release profile of several selected ions such as $Ca^{2+,}$ Na^{+}, Mg^{2+}, K^{+} and PO_4^{3-} in the complete culture media of Saos-2 cells was measured by ICP-OES. This study demonstrated that the combination of GTN-BG is more potent than GTN in inhibiting the proliferation of Saos-2 cells while leaving the viability of HMSC as a control normal cell unaffected. The presence of BG significantly increased the pH values in the culture medium of Saos-2. The BG and GTN-BG culture medium exposed to Saos-2 cells showed a significant increment of Ca^{2+} and Na^{+} ions concentration after 1 to 24 hours of incubation time compared to UT and GTN. The inhibitory effects of GTN in Saos-2 cells were improved when combined with BG, which may result from a better microenvironment. The release of ionic dissolution products from the BG allowed the rise of pHe, which minimised the pHe/pHi ratio, and subsequently affected the progression of the Saos-2 cells.

Keywords**:** Goniothalamin, Bioactive Glass 45S5, Saos-2, pH Values, Ionic Dissolution Products

Introduction

Cancer is a leading cause of death and represents one of the biggest healthcare issues worldwide. Osteosarcoma is a type of primary malignant bone tumour that emerges from the mesenchymal tissue and is characterised by spindle-shaped stromal cells capable of producing bone-like tissues (1). Although the annual incidence is low with 1-3 cases per million people, osteosarcoma is the most common malignant tumour in the skeletal system, especially among children and adolescents. In fact, the propensity of the osteosarcoma cells to metastasise and recur remain the major factor for poor prognosis and treatment failure (2). A multi-modality therapy using a combination of chemotherapy and surgery is the topmost choice to treat patients with high-grade osteosarcoma. However, the chemotherapeutic agents used are cytotoxic in nature to both cancer and normal cells. For this reason, researchers are putting a lot of effort towards developing better strategies to improve the treatment of osteosarcoma and alleviate the severe side effects of conventional treatment (3).

Any compound could be considered a promising alternative to present chemotherapeutic drugs if they have a full range of anticancer activity with less toxicity toward normal cells (4). The interest in plant-derived therapeutics have become significance due to its unique structural complexity in comparison with synthetic molecules (5). One proposed alternative to induce either additive or synergistic effects of the plant-derived compounds is to combine them with biomaterials such as chitosan, collagen, hydroxyapatite, or bioactive glass (6).

Goniothalamin (GTN), a natural styryl-lactone extracted from *Goniothalamus* species has been reported in many studies. The molecule has a potent antitumor effect on several cancer cells, including leukaemia, breast, lung, oral, cervical, colon, ovarian, pancreatic and prostate cancer cells, with less toxicity to normal cells (7). We have previously demonstrated the effectiveness of GTN in killing several human cancer cell lines, including osteosarcoma Saos-2, breast adenocarcinoma MCF-7, breast carcinoma UACC-732, adenocarcinoma alveolar basal epithelial A549 and colorectal adenocarcinoma HT29 cells, with less toxicity towards the normal human mesenchymal stem cells (HMSC) (8).

Bioactive glass (BG) was first discovered by Larry Hench in 1969 with an original BG composed of 45% SiO_2, 24.5% Na_2O, 24.5% CaO and 6% P_2O_5 using the traditional melt method at high temperature (9). The sol-gel method, on the other hand, has the advantages of low processing temperatures and ease of control over textural properties. Furthermore, it has fewer components than melt-derived glasses and may have higher bioactivity and absorbability due to the increased surface area (10). Bioactivity is related to a material's surface area, which affects glass dissolution and apatite formation. Due to its high biocompatibility and bioactivity, it has a wide range of biomedical applications, and are currently used as bone grafts, scaffolds and coating material for dental implants (11, 12). It has been reported that ionic dissolution products such as Si, Ca, and P from BG and other silicate-based glasses products stimulate the expression of several osteoblastic cell genes and angiogenesis *in vitro* and *in vivo*. In addition, the BG ion dissolution also exhibited antibacterial and anti-inflammatory properties (13).

This study was designed to explore the combined effects of GTN and BG (GTN-BG) in Saos-2 cells, focusing on the extracellular changes in the cells' growth media. The assessment of BG degradation in the culture media is essential to predict any effects that may contribute to growth inhibition in Saos-2 cells. Since the release of BG ions may correlate with an increase in the solution's pH; therefore, the pH of Saos-2 cells' growth media in response to treatments was also evaluated.

Materials and methods

Goniothalamin (GTN)

The powdered form of GTN compounds isolated from the roots of *Goniothalamus macrophyllus* was kindly supplied by Prof. Dr. Abdul Manaf Ali from Universiti Sultan Zainal Abidin (UniSZA). It was dissolved in dimethyl sulphoxide (DMSO) (Vivantis, USA) to obtain a stock solution of 10 mg/mL and deposited in aliquots at -20 °C for future usage.

Synthesis of bioactive glass (BG) 45S5

The BG powders were synthesised using the sol-gel method. Briefly, a total of 33.5 mL tetraethyl orthosilicate (TEOS) was added to 50 mL of 1M of Nitric acid and stirred at room temperature for 1 hour. A volume of 2.9 mL triethyl phosphate (TEP), $(C_2H_5)_3PO_4$ and 20.63 g calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$ was added to the solution at 45 minutes intervals. Then, 13.42 g sodium nitrates, $NaNO_3$ was added and left overnight with continuous stirring. The solution was incubated in an oven at 31°C for three days to accelerate gelation. The resulting gel was aged by incubating in the oven for two days at 60 °C and continued the drying process at a temperature of 110 °C for another two days. The dried gel was placed in an alumina cup and was calcined at 700 °C for 3 hours to obtain finer BG powders.

The effects of synthesised BG on the viability of Saos-2 cells

An MTT assay was used to examine the effect of

synthesised BG on Saos-2 cell proliferation at concentrations ranging from 0 to 1 mg/mL. Briefly, 5 × 10^4 cells/mL of Saos-2 cells were seeded overnight in a 96-well plate. Then, the cells were treated with BG from 0 to 1 mg/mL concentration. After 72 hours of incubation, a volume of 20 µL MTT reagent was added and further incubated for 3 hours in a humidified 5% CO_2 incubator at 37^{o}C. The media was discarded, and 100 µL of stop solution was added to each well and further incubated for 1 hour. The absorbance was measured at 570 nm with 630 nm as a reference using a microplate reader.

The dose for the combination treatment of GTN and BG (GTN-BG)

The effect of the combination treatment of GTN and BG (GTN-BG) on Saos-2 cells was assessed by MTT assay as described above. The combination dose for GTN-BG treatment was formulated based on *in vitro* response of GTN and BG. For GTN, the IC_{50} values of GTN in Saos-2 cells for 72 hours; 1.29±0.08 µg/mL obtained from our previous study (8), were employed. Meanwhile, since BG was nontoxic to the treated Saos-2 cells, as none of the IC_{50} values was detected at a concentration from 0 to 1 mg/mL, thus, the median BG concentration at 0.5 mg/mL was picked to be combined with GTN. The dose for combination GTN-BG treatment (GTN; 1.29 µg/mL + BG; 0.5 mg/mL) was also tested on normal HMSC cells as a control.

Evaluation of ionic dissolution and pH measurement in the culture medium of Saos-2 cells

The Saos-2 cells were seeded in a 12-well plate at a concentration of 5 × 10^4 cells/mL and incubated overnight in a 5% CO_2 incubator at 37˚C temperature. Then, the cells were treated with GTN, BG and GTN-BG. After incubation for 1, 24 and 72 hours, the culture media were transferred to a new tube and filtered using a 0.22 µm syringe filter. The pH of the culture media was recorded using a pH meter and stored in a freezer at -80°C for evaluation of ion dissolution by inductively coupled plasma optical emission spectrometry (ICP-OES) (Thermo Scientific, USA). Before ICP-OES analysis, a volume of 1 mL sample was diluted in 15 mL of water (ratio of 1:15). The diluted samples were then filtered through a 0.45 µm nylon filter, aliquoted into 15 mL clean conical tubes and placed on an ASX-520 autosampler. The calibration was done using IV-ICPMS-71 as a standard (Inorganic Ventures, USA). Each measurement was conducted in triplicates and analysed using Thermo Scientific ICAP 7600 Duo (Thermo Scientific, USA) under parameters listed in Table 1, with the same uptake and wash time of 30 seconds. The estimated values of the element obtained were then calculated.

Table 1: The ICP-OES analysis parameters of Thermo Scientific ICAP 7600 Duo, (Thermo Scientific, USA)

View Direction	Radial	Axial
UV Exposure Time	15	15
UV RF Power	1150	1150
UV Neb Gas Flow	0.5	0.5
VIS Exposure Time	5	5
VIS RF Power	1150	1150
VIS Neb Gas Flow	0.5	0.5
Cool Gas Flow Rate	12	12
Aux Gas Flow Rate	0.5	0.5

Statistical analysis

All experiments were performed in three independent experiments, and each data was presented as mean ± standard deviation. For the initial effects of GTN-BG on the proliferation of Saos-2 cells using MTT assay, comparisons of the group were performed by one-way ANOVA, followed by *Tukey*'s post-test for multiple comparisons. Whereas for pH measurement and ion dissolution evaluation, comparisons of the group were made using Two-way ANOVA followed by the *Bonferroni* post-test for multiple comparisons. Significant different between treated and untreated marked as * p<0.05; ** p<0.01; *** p< 0.001. The significant difference between GTN and GTN-BG treatments represent as + p<0.05; ++ p<0.01; +++ p< 0.001 and ns; not significant.

Results

In vitro BG response to Saos-2 cells

As shown in Fig. 1, no IC_{50} values were detected at all concentrations of BG ranging from 0 to 1 mg/mL. The lowest percentage of cell viability was 80% at concentrations of 0.063 mg/mL and 0.5 mg/mL of BG, which were significantly (p<0.001) different from the untreated cells. This is followed by 85% of cell viability (p<0.01), in response to 1 mg/mL of BG. However, the reduction of cell viability at concentrations of 0.125 mg/mL and 0.25 mg/mL were not statistically significant.

The optimal dose for the combination treatment of GTN and BG 45S5 (GTN-BG)

The effects of a combination of GTN and BG; GTN-BG on the proliferation of Saos-2 after 72 hours was assessed by MTT assay in comparison to untreated and treated Saos-2 with BG, GTN, and DOX. As shown in Fig. 2A, the treatment of Saos-2 cells with GTN, GTN-BG and DOX significantly decreased cell proliferation. Compared to single GTN treatment, the inhibitory effects of GTN-BG in Saos-2 cells were significantly higher with $p<0.01$ and $p<0.05$, respectively. The effects of combination GTN-BG using optimal doses of Saos-2 (GTN1; 1.29 µg/mL + BG; 0.5 mg/mL) were also examined on normal HMSC cells' proliferation. As shown in Fig. 2B, the treated HMSC cells with BG, GTN and GTN-BG shows no significant difference compared to untreated HMSC cells. In contrast, treated HMSC cells with positive control DOX significantly ($p<0.001$) reduced the cell viability by almost 50%.

pH measurement in the culture medium

The pH of Saos-2 cells' culture media following different treatments; BG, GTN and GTN-BG, was evaluated at intervals of 1, 24 and 72 hours. As shown in Fig. 3, the pH values decreased over time. The presence of BG, either BG alone or GTN-BG significantly increased the pH values in the culture medium of Saos-2, compared to untreated cells. In comparison to GTN, GTN-BG treatment showed significant ($p<0.001$) increment of pH values at all time intervals.

Evaluation of ionic dissolution

The release profile of several selected ions such as $Ca^{2+,}$ Na^{+}, Mg^{2+}, K^{+} and PO_4^{3-} in the complete culture media of Saos-2 cells was measured by ICP-OES. As shown in Fig. 4, the treated BG and GTN-BG Saos-2 cells' media showed a significant increment of Ca^{2+} and Na^{+} ion concentrations after 1 and 24 hours of incubation time compared to UT and GTN. No further increments were detected after 72 hours. Besides, the concentration of Mg^{2+} ions was also increased after 1 and 72 hours of treatment with BG and GTN-BG. The K^{+} and PO_4^{3-} ion concentrations were slightly increased when BG was present in the culture of Saos-2 cells. However, these increments were not statistically significant.

Discussion

The effectiveness of GTN as a therapeutic agent with a very strong range of cytotoxicity against several cancer cells, including Saos-2, MCF-7, UACC, A549 and HT29 with less toxicity toward the HMSC normal cells has been demonstrated in our previous study (8). In the present study, we found that the inhibitory effects of GTN in Saos-2 cells were enhanced when combined with BG. However, the reason for this enhancement was unclear. We speculate this might be due to several reasons related to BG properties. Since the combination of GTN-BG treatment consists of BG 45S5 as a biomaterial besides GTN as the potential anticancer compound, the assessment of BG degradation in the culture media is essential to predict any effects that may contribute to growth inhibition of Saos-2 cells. Thus, the extracellular changes in the Saos-2 cells' growth media including their pH and ion release pattern were examined in this study. Several ions release profile in the complete culture media of Saos-2 cells was measured by ICP-OES. We found the concentration of Ca^{2+}, $Na^{+,}$ and Mg^{2+} ions were increased in GTN-BG treatment compared to GTN, especially during the initial time of incubation. This result was consistent with Hench's mechanism that describes a rapid exchange between Ca^{2+} and Na^{+} ions in the BG and H^{+} and H_3O^{+} ions coming from the surrounding environment during the initial process of bioglass surface upon contact with body fluid (14). The release of these ions and a silica-rich layer formed on the surface of the glass were correlated with the increase of pH in the solution (9, 14–16).

The pH evaluation profile is particularly essential to study a biomaterial's degradation and bioactivity profile *in vitro*. In this study, the pH of Saos-2 cells' growth media in response to treatments was also evaluated. As expected, the pH values decreased over time as the cells proliferated. However, the antiproliferative activity in the treated GTN and GTN-BG cells resulted in higher pH in their culture media compared to untreated cells. Compared to GTN, the treatment with GTN-BG significantly exhibited higher pH in the cells' growth media due to BG's ion release. Previous studies also reported that releasing ionic dissolution products from the BG, such as $Ca^{2+,}$ Na^{+}, PO_4^{3-}, and Si^{4+} ions, have increased the pH and osmotic pressure in its vicinity. These effects were shown to have an efficient antibacterial effects (17, 18). The role of pH in cancer cells and the cancer microenvironment also has been emphasised in treating cancer cells (19). The extracellular and intracellular pH (pHe and pHi) levels in cancer cells are thought to be acidic and lower than in normal cells. However, recent progress in the measurement of pH in cancer cells has disclosed that the pHi of cancer cells is neutral or even slightly alkaline compared to normal cells (19, 20).

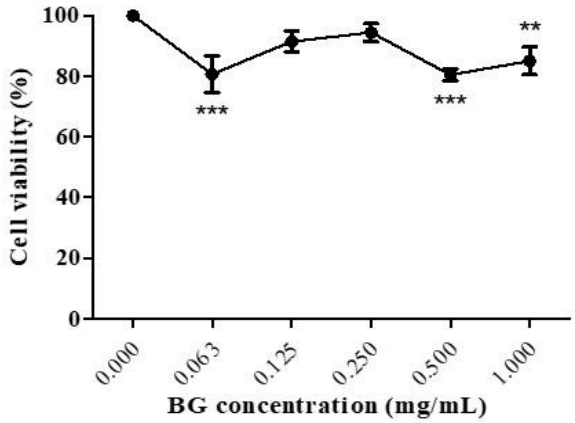

Figure 1: The effect of synthesised BG on Saos-2 cell proliferation at concentrations ranging from 0 to 1 mg/mL, assessed by MTT assay at 72 hours. Data are presented as means±SD, n=3. Comparisons of the group were done using one-way ANOVA, followed by Dunnett's post-test to detect any significant differences (* p<0.05; ** p<0.01; *** p<0.001) between the treated and untreated cells.

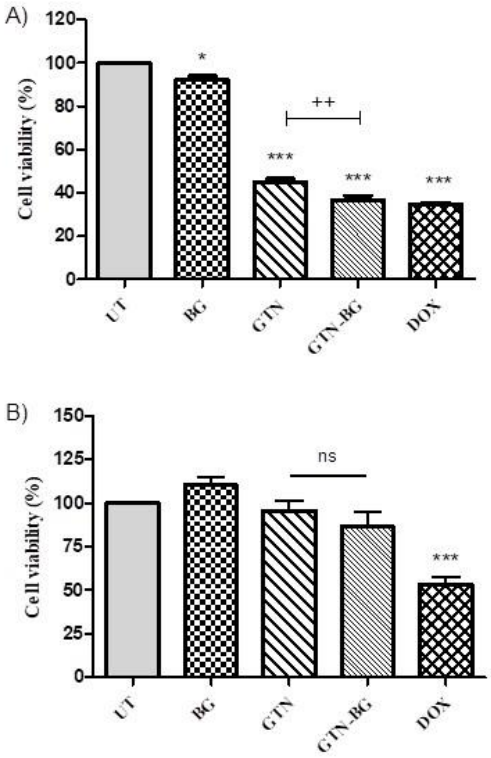

Figure 2: The initial effects of combination GTN and BG; GTN-BG on the proliferation of A) Saos-2 and B) HMSC normal cells, assessed by MTT assay for 72 hours. Data are presented as means±SD, n=3. Comparisons of the group were done using one-way ANOVA, followed by Tukey's post-test for multiple comparisons. Significant differences between treatments and untreated cells are marked as * p<0.05; ** p<0.01; *** p<0.001 whereas the significant differences between GTN and GTN-BG treatments are marked as $^{+}$ p<0.05; $^{++}$ p<0.01; $^{+++}$ p< 0.001; ns not significant.

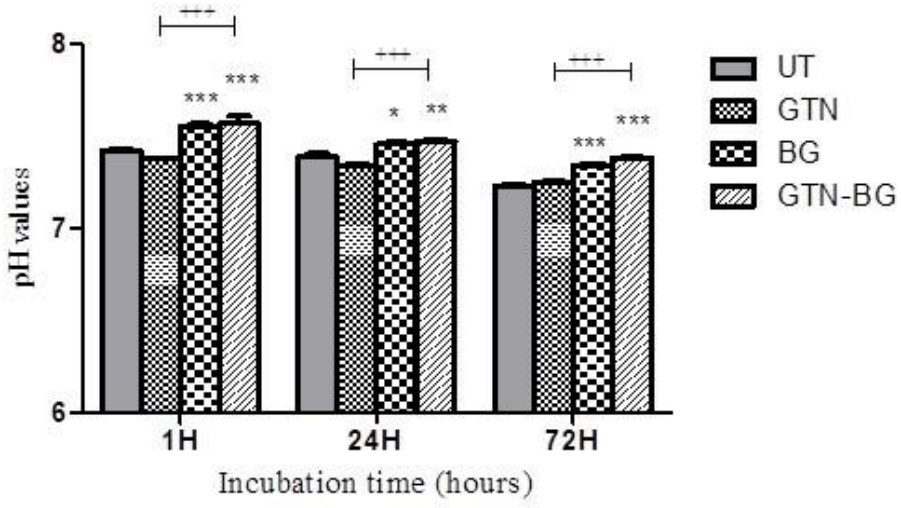

Figure 3: The measurement of culture media pH of Saos-2 cells after treatment with BG, GTN and GTN-BG for up to 72 hours. Data are presented as means±SD, n=3. Comparisons of the group were done by using two-way ANOVA, followed by the Bonferroni post-test for multiple comparisons. Significant differences of treatments compared to untreated cells are marked as * p<0.05; ** p<0.01; *** p<0.001, whereas significant differences between GTN and GTN-BG treatments are marked as + p<0.05; ++ p<0.01; +++ p< 0.001.

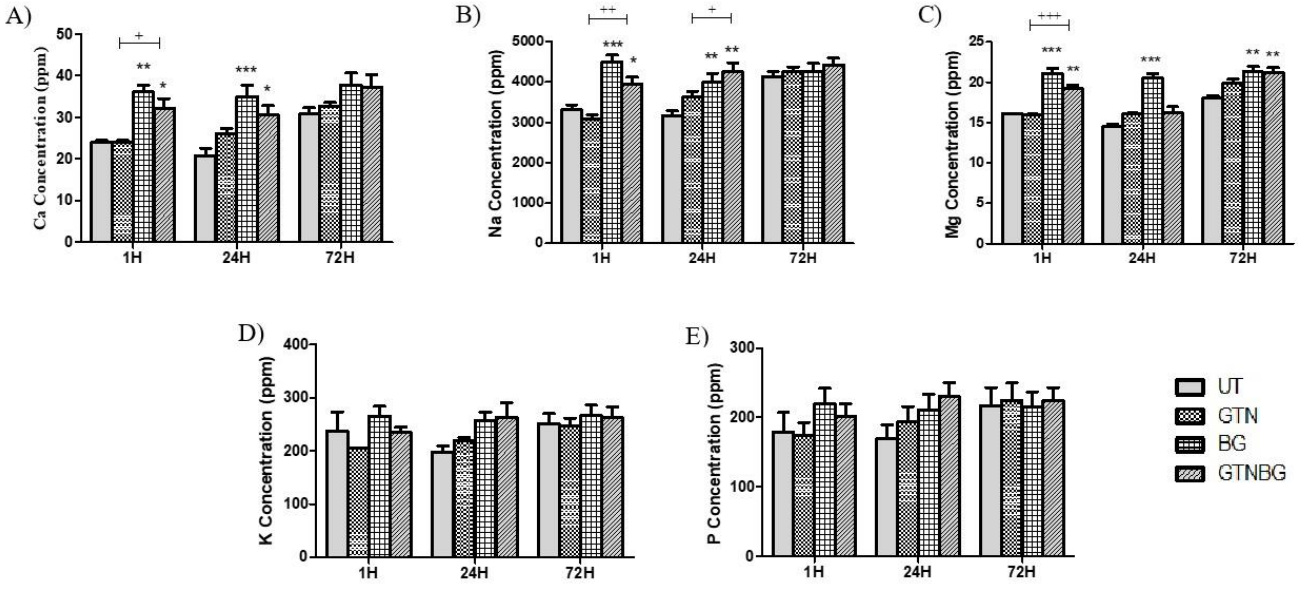

Figure 4: The profiles of A) Ca^{2+}, B) Na^{+}, C) Mg^{2+}, D) K^{+} and E) PO_4^{3-} ion release in the culture media of Saos-2 cells. Data are presented as means±SD, n=3. Comparisons of the group were done by using two-way ANOVA, followed by the Bonferroni post-test for multiple comparisons. Significant differences of treatments compared to untreated cells are marked as * p<0.05; ** p<0.01; *** p<0.001, whereas significant differences between GTN and GTN-BG treatments are marked as + p<0.05; ++ p<0.01; +++ p<0.001.

A reverse pH gradient manifested by extracellular acidosis and intracellular alkalisation is a hallmark of cancer metabolism. The abnormally high ratio of pHe/pHi in cancer cells is due to the high rate of glycolysis which produces numerous acidic products that can promote migration, invasion and metastasis of cancer cells (21). Dysregulation of cellular pH provides potential opportunities for therapeutic intervention. Increasing the pHe can be considered as one strategy to inhibit the progression of tumour cells (19-20, 22). In this study, the ratio of pHe/pHi in Saos-2 cells has been minimised due to the rise of pHe during the release of ionic dissolution products from the BG and thus, might be one of the explanations for the enhancement of antiproliferative activity in GTN-BG compared to GTN treatment.

The preliminary step has been already done in this study to indicate positive outcome of GTN-BG on Saos-2 cells. Nevertheless, an extensive and systematic investigations on the biological effects of GTN-BG using broad range of optimum dosages are necessary to conclude its overall cytotoxicity mechanisms. In addition, *in vivo* studies using relevant animal models are important to evaluate the fundamental mechanisms underlying cancer progression which is associated with the ionic dissolution products in the microenvironment.

Conclusion

The inhibitory effects of GTN-BG were higher than in GTN-treated Saos-2 cells with less toxicity towards HMSC normal cells. In comparison to GTN, the enhanced inhibitory effects of GTN-BG is due to a better microenvironment provided, as the release of ionic dissolution products from the BG allowed the rise of pHe, minimised the pHe/pHi ratio, and subsequently affected the proliferation of the Saos-2 cells.

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Competing interests

The authors have no conflict of interest associated with this study.

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