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Hydrogen sulfide donor GYY4137 suppresses proliferation of human colorectal cancer Caco-2 cells by inducing both cell cycle arrest and cell death

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ABSTRACT

Conflicting data regarding the ability of hydrogen sulfide (H_2S), which reaches high levels in the large intestine owing to biosynthesis in the intestinal cells and intestinal bacteria, to promote or inhibit colorectal cancer cell proliferation have been reported recently. In the present study, the effect of H_2S on the proliferation of the human colorectal cancer cell line Caco-2 was examined by using the H_2S donor GYY4137. At concentrations of 0.5 mM and 1.0 mM, GYY4137 significantly inhibited Caco-2 cell viability. Cell cycle analysis, and apoptosis and necrosis detection revealed that the anti-proliferative effect of GYY4137 was partially attributable to the induction of S-G_2/M cell cycle arrest, apoptosis and necrosis. These results suggest that H_2S has the potential to suppress human colorectal cancer cell proliferation by influencing both cell cycle and cell death.

1. Introduction

Colorectal cancer is one of the leading causes of death worldwide, being responsible for approximately 10% of total cancer-related mortality [1]. About 3–5% of colorectal cancers may be due to inherited genetic defects, and up to 25% of patients may have some degree of hereditary predisposition for this disease, although the majority of colorectal cancers occur in a sporadic manner, in the absence of documented family history [2].

Hydrogen sulfide (H_2S) is synthesized naturally from cysteine by several enzymes, including cystathionine γ-lyase, cystathionine β-synthetase, and 3-mercaptosulfurtransferase, in a wide range of mammalian and non-mammalian cells both *in vitro* and *in vivo*. In the last two decades, numerous physiological and pathophysiological roles have been proposed for this gas along with a plethora of cellular and molecular targets, including a range of ion channels, enzymes, and transcription factors [3]. In the large intestine, H_2S is mainly produced by endogenous sulfate-reducing bacteria, and its concentration reaches up to 3.4 mM in human colon [3, 4, 5]. It has also been reported that in colon cancer patients, the amount of H_2S is increased, because the expression of H_2S-metabolizing enzymes is reduced [6, 7]. Moreover, fecal sulfide level is elevated in patients with ulcerative colitis, a condition associated with an increased risk of colon cancer [8].

To date, many studies have been undertaken using sulfide salts such as sodium hydrosulfide (NaHS), which release H_2S instantaneously into aqueous solution. However, the release of endogenous H_2S from cells is likely to occur in lesser amounts and at a much slower rate than that from sulfide salts, and therefore, NaHS may not accurately mimic the biological effects of naturally produced H_2S. Recognizing the need for organic molecules capable of releasing H_2S over extended periods of time, Li et al. [9] reported that morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GYY4137) releases H_2S slowly both *in vitro* and *in vivo*.

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The present study was undertaken to investigate the effect of GYY4137, a slow-releasing H_2S donor, on the proliferation of the colorectal cancer cell line Caco-2. Further, a possible mechanism of the H_2S effect was evaluated by analyzing cell cycle and cell death characteristics.

2. Materials and methods

2.1. Materials

GYY4137 was obtained from Dojindo Molecular Technologies, Inc. (Rockville, MD, USA). NaHS was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Annexin V-FLUOS Staining Kit and *In Situ* Cell Death Detection Kit, fluorescein were purchased from Roche Diagnostics (Indianapolis, IN, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ribonuclease A (RNase A), and propidium iodide (PI) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Measurement of H_2S

After incubation with GYY4137 (1.0 mM, 100 μL) or NaHS (1.0 mM, 100 μL) in Minimum Essential Medium (MEM; Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and 1% non-essential amino acids (Life Technologies Corporation, Carlsbad, CA, USA), aliquots were mixed with a solution of 0.85% zinc acetate (w/v) and 3% NaOH (1:1 ratio, 100 μL). Methylene blue was then formed by the addition of N,N-dimethyl-p-phenylenediamine-dihydrochloride dye and $FeCl_3$ at final concentrations of 2.5 and 3.3 mM, respectively, and absorbance was subsequently monitored at 670 nm. The concentration of H_2S was determined using a standard curve of NaHS (0–400 μM).

2.3. Cell culture

Caco-2 human colon cancer cell line was purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and cultured in MEM supplemented with 10% fetal bovine serum and 1% non-essential amino acids. The cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.4. Cell viability assay

Cell viability was measured by the MTT assay, as described previously [10, 11]. Briefly, the cells at a density of 2.5×10^4 cells/2 mL/9.5 cm^2 well were incubated with the test reagents for 72 h. After incubation, the medium was removed, and the cells were incubated with 1.1 mL of MTT solution (0.1 mL of 5 mg/mL MTT in 1 mL of the medium) for 4 h. The product was eluted from the cells by the addition of 20% sodium dodecyl sulphate/0.01 M HCl, and absorbance at 595 nm was determined using an SH-1200 Lab microplate reader (Corona Electric Co., Ltd, Ibaraki, Japan). Cell viability was calculated according to the following equation: cell viability (%) = (absorbance of experiment group/absorbance of control group) × 100.

2.5. Cell cycle analysis

Cell cycle analysis was performed by flow cytometry as reported previously [10, 11]. Briefly, the cells at a density of 1.0×10^6 cells/28 cm^2 dish were incubated with the test reagents for 72 h and then collected by centrifugation (4 °C, 200 × *g*, 5 min). The pellet was fixed with 70% ethanol cooled at −20 °C on ice for 30 min. Following fixation, the cells were incubated with 100 μg/mL RNase A at 37 °C for 30 min. The cells were treated with PI (50 μg/mL) in a dark place on ice for 30 min. The samples were filtrated through a nylon mesh (35 μm) and examined in a FACScan™ flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, US).

2.6. Determination of cell apoptosis and/or necrosis

DNA fragmentation in cell nucleus, an indicator of apoptosis, was detected by using an *in situ* Cell Death Detection Kit and fluorescein, and confocal laser scanning microscope (Carl Zeiss Co., Ltd., LSM-510; excitation at 495 nm and emission at 530 nm), as reported previously [12]. Briefly, the cells at a density of 3.0×10^5 cells/0.8 cm^2 of Nunc™ Lab-Tek™ Chamber Slide (Thermofisher Scientific K.K., Tokyo, Japan) were incubated with the test reagents for 48 h. Blue coloring indicates cell nuclei stained by 4′,6-diamidino-2-phenylindole (DAPI). TUNEL-positive nuclei (apoptotic cells) were visualized by green coloring. Clear light blue coloring (a mixture of blue coloring and green coloring) shows DNA fragmentation in the nuclei.

In addition, apoptosis or necrosis was detected by flow cytometry using an annexin V-fluorescein staining kit. Briefly, the cells were incubated with the test reagents at a density of 1.0×10^6 cells/28 cm^2 dish for 48 h, and then collected by centrifugation. The cell pellets were incubated with staining solution containing annexin V-fluorescein and PI at room temperature for 15 min. After adequate dilution according to the cell density, the samples were filtrated through a nylon mesh (35 μm), and subjected to a FACS Aria™ III flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ), as reported previously [13].

2.7. Statistics

Results are the means ± SEM. The significance of differences between two groups was assessed using the Student's *t* test, and differences between multiple groups were assessed by one-way analysis of variance (ANOVA), followed by Scheffe's multiple range test. *P* values less than 0.05 were considered significant.

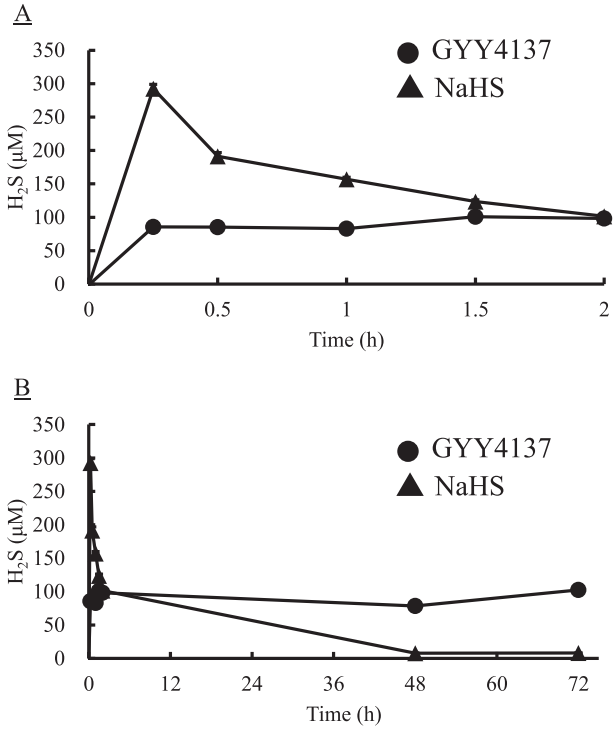

Fig. 1. Release of H_2S by GYY4137 (1.0 mM, ●) and NaHS (1.0 mM, ▲) into culture medium. (A) H_2S release was determined at time intervals up to 2 h. (B) H_2S release was determined at time intervals up to 72 h. Data are expressed as the mean ± SEM ($n = 4$).

Table 1
Effects of GYY4137 and NaHS on the proliferation of Caco-2 cells.

Treatment		Cell viability (% of control)
Control		100.0 ± 3.4
GYY4137	0.5 mM	69.1 ± 5.0$^{\alpha}$
	1.0 mM	47.1 ± 4.3$^{\alpha\beta}$
NaHS	0.5 mM	76.6 ± 4.7$^{\alpha}$
	1.0 mM	69.0 ± 4.2$^{\alpha}$

Caco-2 cells were treated with GYY4137 or NaHS for 72 h. Proliferation was assayed by the MTT assay. Data are expressed as the mean ± SEM ($n = 9$). $^{\alpha}P <$ 0.01 vs. Control; $^{\beta}P<0.01$ vs. 1.0 mM NaHS.

3. Results

3.1. Release of H_2S from GYY4137

As shown in Fig. 1, incubation in culture medium containing either 1 mM GYY4137 or 1 mM NaHS resulted in the release of micromolar concentrations of H_2S detected in collected aliquots by the methylene blue formation assay. Release of H_2S from NaHS was rapid, peaking at or before 20 min (Fig. 1A) and declining to undetectable levels by 48 h (Fig. 1B). In contrast, peak H_2S release from GYY4137 was much lower (about one third of that observed with NaHS) (Fig. 1A), but it was sustained for up to 72 h (Fig. 1B). A similar result was observed by Lee et al. [14]. The results illustrated in Fig. 1 confirm that NaHS and GYY4137 are rapid-releasing and slow-releasing H_2S donors, respectively.

3.2. GYY4137 suppresses the proliferation of Caco-2 cells more potently than NaHS

Table 1 shows effects of GYY4137 and NaHS at concentrations of 0.5 and 1.0 mM on Caco-2 cell viability. Caco-2 cells were incubated with either GYY4137 or NaHS for 72 h and then, changes in cell viability were measured by the MTT assay. GYY4137 significantly decreased cell viability both at 0.5 mM and 1.0 mM by 30.9% and 52.9%, respectively ($P < 0.01$ vs. Control). Although NaHS suppressed the cell growth (0.5 and 1.0 mM, 23.4 and 31.0% inhibition), the inhibitory efficacy of 1.0 mM GYY4137 was significantly more than that of 1.0 mM NaHS. These results mean that GYY4137 inhibited Caco-2 cell growth more potently than NaHS.

3.3. GYY4137 induces S-G2/M phase arrest in Caco-2 cells

To determine whether GYY4137-induced cell growth inhibition involved cell cycle changes, we examined distribution of cells in cell cycle phases by flow cytometry (Fig. 2). The treatment of Caco-2 cells with 0.5 and 1.0 mM GYY4137 for 72 h induced an accumulation of cells in the S and G2/M phases of cell cycle. Concomitantly with these increases in the population of cells in the S and G2/M phases, a decrease in the population of cells in the G0/G1 phase was observed. This result implies that GYY4137 induced an arrest of Caco-2 cells in the S-G2/M phase.

3.4. GYY4137 induces apoptosis and necrosis in Caco-2 cells

To test whether the GYY4137-induced inhibition of Caco-2 cell

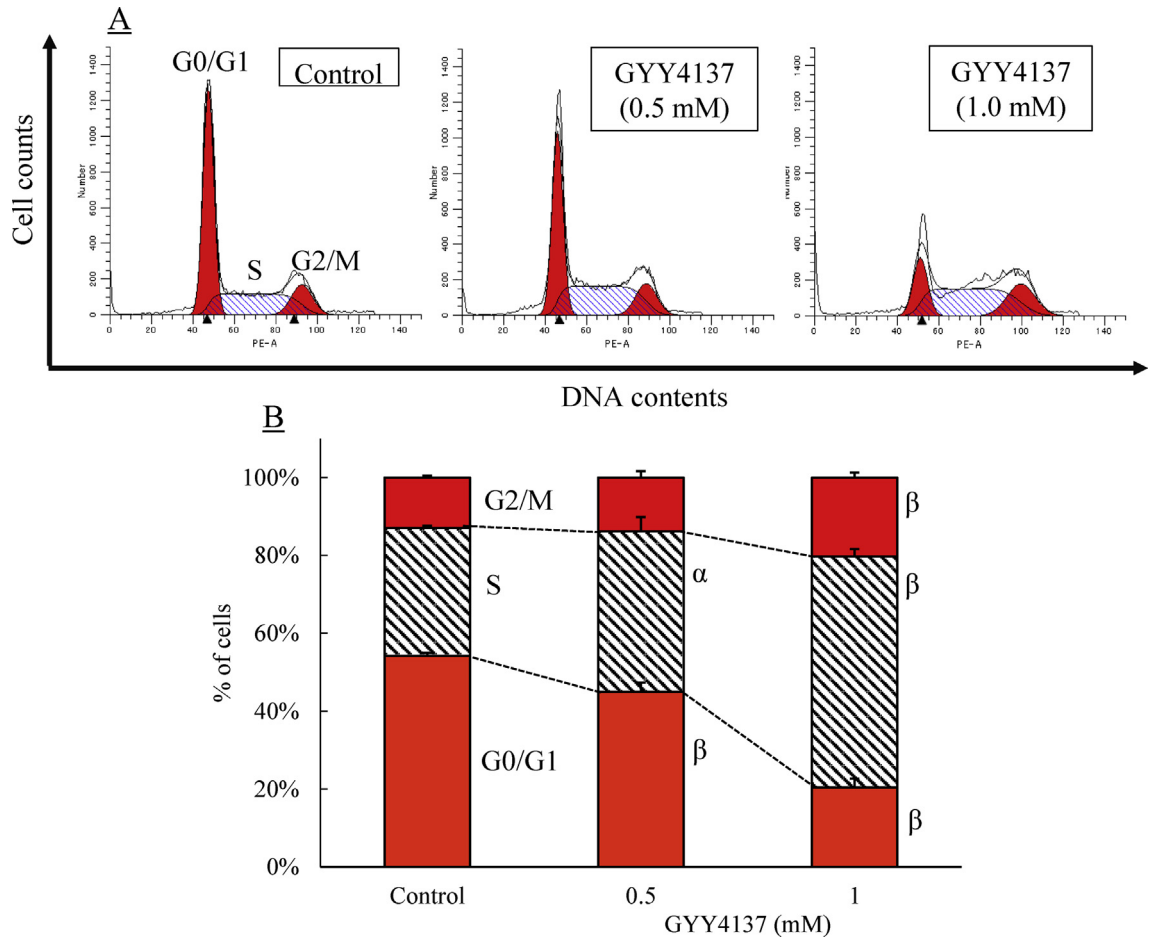

Fig. 2. Effect of GYY4137 on the distribution of Caco-2 cells in different phases of cell cycle. Caco-2 cells were treated with 0.5 and 1.0 mM GYY4137 for 72 h. (A) Representative flow cytometry charts. (B) The effect of 0.5 and 1.0 mM GYY4137 on the percentages of Caco-2 cells in the G0/G1, S, and G2/M phases. Data are expressed as the mean ± SEM ($n = 7$). $^{\alpha}P < 0.05$, $^{\beta}P < 0.01$; significantly different from the corresponding value in the cells that were not exposed to GYY4137.

proliferation was due to apoptosis and/or necrosis, apoptotic cells were visualized by the TUNEL assay, using a confocal laser scanning microscope (Fig. 3). Blue coloring indicates cell nuclei stained by DAPI. TUNEL-positive nuclei were visualized by green coloring. Clear light blue coloring (a mixture of blue coloring and green coloring) shows DNA fragmentation in the nuclei, as indicated by arrows. The addition of 0.5 μM actinomycin D as positive control caused DNA fragmentation in the nuclei, i.e., hallmarks of apoptotic cells. Similarly to actinomycin D, 0.5 mM GYY4137 also increased the number of apoptotic cells in a small extent.

Fig. 4 also illustrates the percentages of apoptotic or necrotic cells, measured by the annexin V-fluorescein/PI assay using flow cytometry, when Caco-2 cells were exposed to 0.5 and 1.0 mM GYY4137 at 48 h. Positive staining with annexin V-fluorescein correlates with a loss of membrane polarity (apoptosis). In contrast, PI can only enter cells after the complete loss of membrane integrity (necrosis). The fraction of the cell population in different quadrants was analyzed using quadrant statistics [13]. Cells in the upper left quadrant [a: annexin V(–)/PI(+)] represented necrotic cells. Cells in the lower right quadrant [d: annexin V(+)/PI(–)] represented early apoptotic cells, and cells in the upper right quadrant [b: annexin V(+)/PI(+)] represented late apoptotic cells. Analytic data of the control, actinomycin D (0.5 μM), and 0.5 and 1.0 mM GYY4137 is shown Fig. 4A. The experiment using actinomycin D (0.5 μM), which is a known inducer of apoptosis, verified the validity of the cell analysis; it significantly increased both the early apoptotic and late apoptotic cell population [Fig. 4B(b,d)]. The treatment of Caco-2 cells with 1.0 mM GYY4137 induced a significant increase in both the early apoptotic and late apoptotic cell population [Fig. 4B(b,d)] in the same way as actinomycin D. Additionally it enhanced the necrotic cell population [Fig. 4B(a)]. The results imply that GYY4137 has the potential to induce both apoptosis and necrosis in Caco-2 cells.

4. Discussion

There have been conflicting reports regarding the role of H_2S in colorectal cancer. Therefore, we have examined here the effect of H_2S on the proliferation of human colorectal cancer Caco-2 cells and showed that the H_2S donor GYY4137 significantly attenuated Caco-2 cell

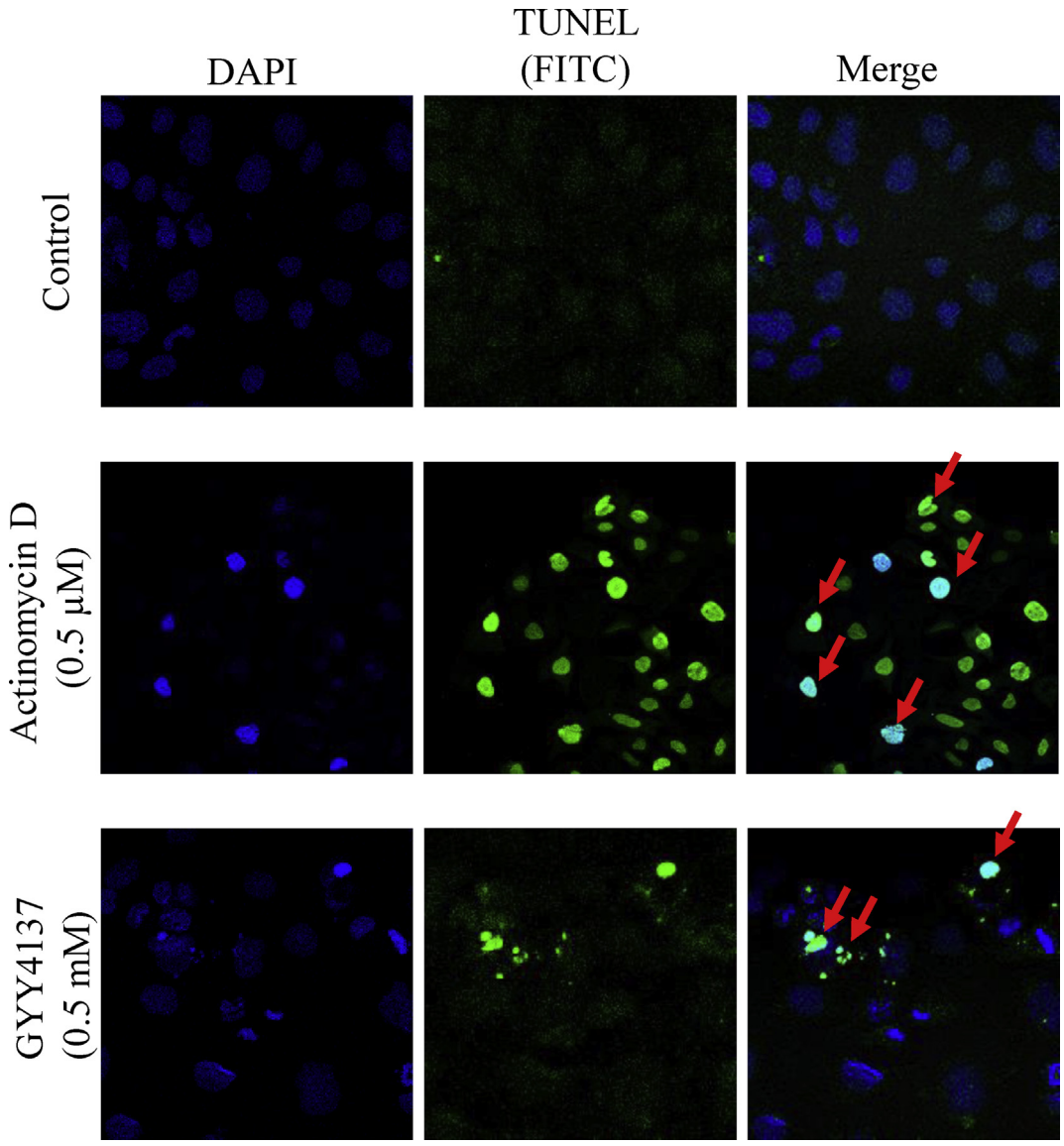

Fig. 3. Fluorescence image of DNA fragmentation in Caco-2 cells treated with GYY4137. Caco-2 cells were treated with 0.5 mM GYY4137 for 72 h. Apoptotic cells were visualized by the TUNEL assays using a confocal laser scanning microscope. Blue coloring indicates cell nuclei stained by DAPI. TUNEL-positive nuclei were visualized by green coloring. Clear light blue coloring (a mixture of blue coloring and green coloring) shows DNA fragmentation in the nuclei, as indicated by arrows. Data were collected from at least 10 random sections per sample. The data are representative of 3 experiments.

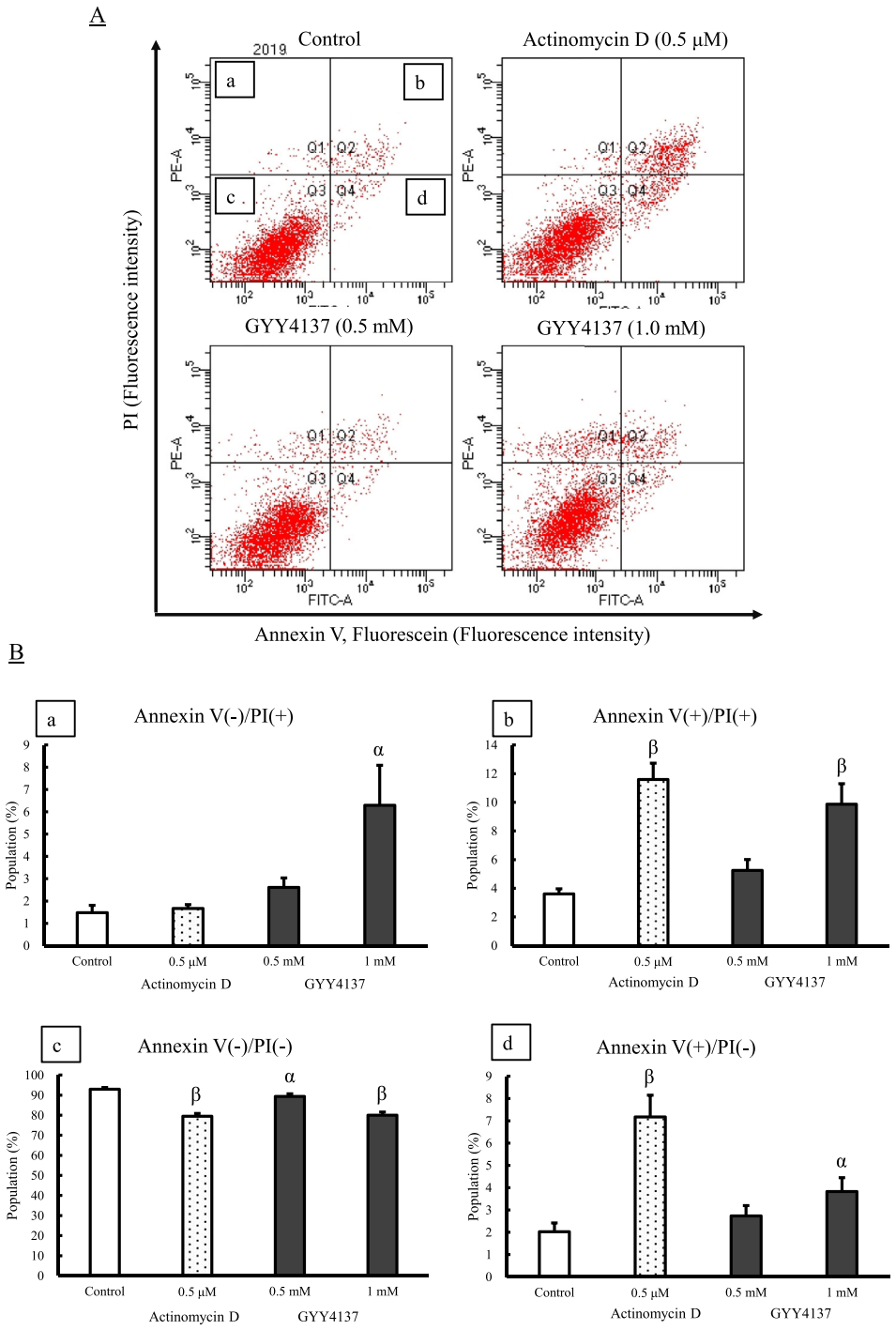

Fig. 4. Effect of GYY4137 on apoptosis or necrosis in Caco-2 cells. Caco-2 cells were treated with 0.5 and 1.0 mM GYY4137 for 48 h. Apoptotic or necrotic cells were measured by the annexin V-fluorescein/PI using a flow cytometry. Data are expressed as the mean ± SEM ($n = 4$). $^{\alpha}P < 0.05$, $^{\beta}P < 0.01$; significantly different from the corresponding value in the cells that were not exposed to GYY4137.

viability in part due to the induction of S-G_2/M cell cycle arrest, apoptosis, and necrosis.

As shown in Fig. 1 and Table 1, the suppression of Caco-2 cell proliferation by H_2S at a low concentration following the exposure to GYY4137 occurred over a period of several days under the indicated experimental conditions, i.e., at a comparatively slow rate. In contrast, the exposure of cells to NaHS generated H_2S at a higher concentration, and Caco-2 cell proliferation was inhibited over a shorter time frame (fast rate). Thus, it is likely that both the duration of exposure to H_2S and its concentration are critical in determining the ability of this gas to inhibit cell proliferation. It is generally accepted that the release of endogenous H_2S from cells occurs in low concentrations, and therefore, the consequences of H_2S generation at the lower rate after the exposure to GYY4137 more closely mimic biological effects of naturally produced H_2S.

Next, some experiments were undertaken to determine the mechanism by which GYY4137 reduced Caco-2 cell proliferation. Flow cytometry analysis of the distribution of treated cultured cells in different phases of cell cycle revealed that both 0.5 and 1.0 mM GYY4137 induced S-G2/M phase cell cycle arrest with an accompanying decrease in the

number of cells in the G0/G1 phase (Fig. 2). This confirmed that GYY4137 inhibited DNA synthesis and induced a block at the S-G2/M boundary. The confocal laser scanning microscopy and flow cytometry observations using of the TUNEL and annexin V-fluorescein/PI assays, respectively (Figs. 3 and 4) revealed that GYY4137 at 0.5 or 1.0 mM induced both apoptosis and necrosis in Caco-2 cells.

Caco-2 cells are one of cancer cell lines that are deficient in p53 protein expression [15, 16]. However, Tsai et al [17] have reported that, independent of p53, activation of extracellular signal-regulated kinase (ERK) leads to both cell cycle arrest and apoptotic cell death via upregulation of p21 and downregulation of Bcl-2, respectively. Yang et al. [18, 19] have also reported that treatment of human aorta smooth muscle cells with H_2S elicits an increase in ERK signaling. Therefore, a certain role of ERK activation in inhibiting Caco-2 cell proliferation by GYY4137 may be one possible mechanism.

There have been disparate observations of the effect of H_2S on colorectal cancer cell growth *in vitro*. Rose et al. [20] have reported that 0.25–1.0 mM NaHS protected HCT-116 colorectal cancer cells from apoptosis caused by the chemoprotective agent β-phenylethyl isothiocyanate. Cai et al. [21] have also shown that NaHS at 0.2–1.0 mM promoted the proliferation of human colorectal cancer cell lines HCT-116 and SW480. In contrast, Cao et al. [22] reported that NaHS at millimolar concentrations inhibited cell growth of WiDr colorectal cancer cells. Wu et al. [23] further showed that NaHS at 0.4–1.0 mM inhibited growth of the colorectal cancer cell lines HT-29, SW1116, and HCT-116 via G1 phase cell cycle arrest. In the present study, we showed that GYY4137 inhibited Caco-2 cell growth by the induction of both S-G2/M cell cycle arrest and cell death. These discrepant observations are difficult to reconcile. However, one explanation may be given for the choice of colon cancer cell line: we used Caco-2 cells in the present study, whereas cancer cell lines other than Caco-2 were used in the previous studies [20, 21, 22, 23]. Further studies are needed for the ultimate reconciliation of these two opposite observations.

In conclusion, we found that GYY4137 exhibited anti-proliferative activity against Caco-2 cells. We propose that GYY4137 breaks down slowly to yield H_2S, which by simultaneously inducing cell cycle arrest, apoptosis and necrosis, inhibits cell growth. The previous findings by Lee et al. [14] that cancer cells, but not non-cancer cells, can be killed selectively when exposed to relatively small amounts of H_2S over a relatively long time period support the significance of the present observations. We believe that our study makes a significant contribution to the literature because, to the best of our knowledge, this is the first time that GYY4137 was shown to reduce Caco-2 cell growth, possibly by inducing both S-G2/M cell cycle arrest, apoptosis and necrosis.

Declarations

Author contribution statement

Satoru Sakuma: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Saaya Minamino, Maya Takase, Yoshitaka Ishiyama, Hiroyuki Hosokura, Yukino Ikeda: Performed the experiments.

Tetsuya Kohda: Contributed reagents, materials, analysis tools or data.

Yohko Fujimoto: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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