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Bucillamine induces glutathione biosynthesis via activation of the transcription factor Nrf2

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ARTICLE INFO

Article history:
Received 30 March 2006
Accepted 17 May 2006

Keywords:
Bucillamine
Glutamate–cysteine ligase (GCL)
Multidrug-resistance-associated protein 2(Mrp2/MRP2)
Glutathione (GSH)
Nuclear factor-erythroid 2 p45-related factor (Nrf2)

Abbreviations:
ARE, antioxidant-response element
DMEM, Dulbecco's modified Eagle's medium
DTT, dithiothreitol
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GCL, glutamate–cysteine ligase
GCLC, GCL catalytic subunit
GSH, glutathione
GSSG, oxidized glutathione
GST, glutathione S-transferase
MRP, multidrug-resistance-associated protein
Nrf2, nuclear factor-erythroid 2 p45-related factor
PMSF, phenylmethylsulphonyl fluoride
ROS, reactive oxygen species
RT-PCR, reverse-transcription polymerase chain reaction

ABSTRACT

The properties of bucillamine, a synthetic antioxidant, have been attributed mainly to the donation of thiol groups to glutathione (GSH). We recently demonstrated that glutamate–cysteine ligase catalytic subunit (GCLC), the rate-limiting enzyme of GSH biosynthesis, and the multidrug-resistance-associated protein 2 (Mrp2/MRP2) are coordinately induced in response to xenobiotic through the activation of the antioxidant-response element (ARE) by nuclear factor-erythroid 2 p45-related factor (Nrf2). We tested the hypothesis that bucillamine and its oxidized metabolite SA 981 also activate the Nrf2 pathway, thereby increasing glutathione biosynthesis in human HepG2 and murine Hepa 1–6 hepatoma cell lines, through the induction of the GCLC enzyme as well as the Mrp2/MRP2 transporter, which mediates the excretion of glutathione and its conjugates from hepatocytes. Both bucillamine and SA 981 produced a significant dose-dependent increase in the mRNA levels of Mrp2/MRP2 and GCLC after 24 h. The levels of the transcription factor Nrf2 in the nuclei were maximal at 3 h, remained elevated at 6 h, and decreased to control values at 24 h in both cell lines. Moreover, both bucillamine and SA 981 significantly increased the expressions of Mrp2/MRP2 and GCLC proteins in both cell lines. Finally, in both cell lines, bucillamine and SA 981 increased the GSH content two- to three-fold. These results demonstrate that bucillamine and SA 981 activate the ARE–ARE pathway increasing the expression of ARE-driven genes such as those of GCLC and Mrp2/MRP2. The role of bucillamine as a chemopreventive agent against cancer remains to be elucidated.

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.05.011

1. Introduction

Bucillamine [*N*-(2-mercapto-2-methylpropionyl)-L-cysteine] is a synthetic cysteine-derivative antioxidant that is used clinically in Japan and Korea for the treatment of rheumatoid arthritis [1]. This drug contains two thiol groups per molecule and is more potent than other antioxidants such as the cysteine-derived agents *N*-acetylcysteine and *N*-(2-mercaptopropionyl)glycine, both of which contain only a single donatable thiol group [2,3]. The tripeptide glutathione (GSH), the most abundant antioxidant in cells, is required for the protection against oxidative damage, detoxification of endogenous and exogenous molecules, and also the regulation of gene expression [4,5]. The biosynthesis of GSH involves two enzymatic reactions catalyzed by the rate-limiting enzyme glutamate–cysteine ligase (GCL; also named γ-glutamylcysteine synthetase) and GSH synthetase [6], with cysteine being the rate-limiting intracellular precursor of GSH [7]. Under conditions of oxidative stress and GSH depletion, bucillamine can be rapidly transported into cells to restore intracellular GSH levels-given the similarity of the chemical structures of cysteine and bucillamine, this probably involves the same pathway(s) utilized by cysteine (Fig. 1) [3,8]. The antioxidant property of bucillamine has been attributed to the donation of thiol groups to glutathione rather than to the *de novo* synthesis of GSH [3].

Several compounds such as flavonoids, phenolic antioxidants, isothiocyanates, and 1,2-dithio-3-thiones are inducers of GCL and Phase II detoxifying enzymes such as the family of glutathione *S*-transferases (GSTs), which are involved in the conjugation of various electrophilic compounds and reactive oxygen species (ROS) with GSH [9–11]. The induction of these enzymes is mediated by the antioxidant-response element (ARE) [12], a *cis*-acting sequence found in the 5′-flanking region of genes such as Gstal [13], NAD(P)H:quinone oxidoreductase [14], and the catalytic subunit of GCL (GCLC) [15]. The factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), in combination with a small Maf protein, mediates transcriptional activation of genes via the ARE [16,17]. Accumulated data from studies of Nrf2-knockout mice and overexpression of Nrf2 in human hepatoma cells have established that Nrf2 is an essential ARE-binding factor involved in both constitutive and inducible gene expression [18,19].

The Mrp2/MRP2 pump, also named ABCC2, is a member of the family of ABC (ATP-binding cassette) transporters that mediates the excretion of GSH and glutathione disulfide, as well as that of endo-and xenobiotics conjugated with GSH, glucuronate, or sulfate [20–23]. We have recently demonstrated that the transcription factor Nrf2 appears to regulate Mrp2 (ABCC2) gene expression via an ARE located at the proximal region of its promoter in response to exposure to xenobiotics [24]. We also demonstrated that the enzymes responsible for the formation of GSH conjugates (GCL and GSTs) and Mrp2 are coordinately induced in response to xenobiotics through the activation of the ARE-Nrf2 cellular detoxification pathway [24]. This lead us to the hypothesis that Nrf2 mediates the coordinated regulation of glutathione biosynthesis, along with phase II (GST) and phase III (Mrp2) drug metabolism [24].

It has been demonstrated that bucillamine and its oxidized metabolite SA 981 increase the mRNA and proteins levels of the vascular endothelial growth factor in dermal fibroblasts from humans with systemic sclerosis [25]. The induction was mediated by activation of the NF-kB and SP-1 pathways [25]. However, the molecular mechanism of the beneficial action of bucillamine has not been fully elucidated.

In this context, we tested the hypothesis that bucillamine and SA 981 may also activate the Nrf2 pathway-thereby increasing glutathione biosynthesis in human and murine hepatoma cell lines-through the induction of the GCLC gene and its encoded enzyme, as well as of the Mrp2/MRP2 transporter, which mediates the excretion of glutathione and its conjugates from hepatocytes.

2. Materials and methods

2.1. Materials

Bucillamine and SA 981 were a generous gift from Santen (Osaka, Japan). Murine Hepa 1–6 and human HepG2 hepatoma cells were purchased from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's culture medium (DMEM) and 10% (v/v) fetal bovine serum was purchased from Invitrogen (Buffalo, NY, USA). Penicillin G, streptomycin sulfate, and fungizone were obtained from Gibco (Langley, OK, USA). Nylon filters were obtained from NEN Research Products (Boston, MA, USA), and the Random Primer Labelling system was obtained from Promega (Madison, WI, USA). The Bradford kit used to assess protein concentration was obtained from Bio-Rad Laboratories (Hercules, CA, USA). The human GCLC polyclonal antibody was a generous gift from Dr. Lesley McLellan (Biomedical Research Centre, University of Dundee, UK). The Mrp2/MRP2 monoclonal antibody M2 III-5 was provided by R.P. Oude Elferink and A.K. Groen (Academic Medical Centre, Amsterdam, The Netherlands). The anti-Nrf2 antibody (sc-722X) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the anti-β-actin monoclonal antibody (ab8226-100) was obtained from Abeam (Cambridge, MA, USA)

2.2. Cell cultures and drugs

Murine Hepa 1–6 and human HepG2 hepatoblastoma cells were cultured in DMEM supplemented with 10% (v/v) fetal

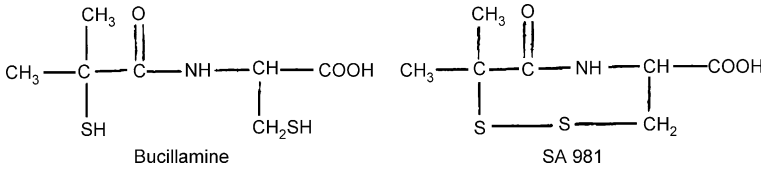

Fig. 1 – Chemical structures of bucillamine and the intramolecular disulphide of bucillamine (SA 981).

bovine serum, 100 units/ml penicillin G, 100 units/ml streptomycin sulfate, and 0.85% fungizone. Bucillamine and SA 981 were dissolved in DMEM and diluted to final concentrations of 0.2, 0.5, and 1.0 mg/ml. Cells were seeded in six-well plates 24 h before the treatment, at which time the culture medium was replaced with fresh complete medium containing the final concentration of each drug. Cells were harvested at 24 h after the addition of bucillamine or SA 981.

2.3. RNA isolation and Northern blot analysis

Total RNA was isolated from cells using the guanidinium–phenol method [26], resolved (10 μg/lane) on a 1.5% agarose and 2.2 M formaldehyde gel, and transferred to nylon filters. The membranes were hybridized with mouse or human ^{32}P-labeled DNA probes as described previously [27]. The DNA probes were obtained through a reverse-transcription polymerase chain reaction (RT-PCR) using specific primers from the cDNA of mouse liver or HepG2 cells, and radiolabeled by the Random Prime Labelling system. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to normalize the RNA loading. The specific primers used are listed in Table 1.

2.4. Western blot analysis

Proteins were separated by SDS-PAGE, with the total membrane and cytoplasmic fractions obtained from control and treated cells as follows. Cells were washed with PBS and scraped from the dishes with ice-cold buffer A (0.25 M sucrose, 10 mM Tris (pH 7.6), and 0.2 mM PMSF). The homogenates were centrifuged at 4 °C for 10 min at 3000 × *g*, and then the supernatant was ultracentrifuged at 100,000 × *g*, for 1 h. The obtained supernatant was stored as the cytoplasmic fraction. The pellet was resuspended in buffer A with 2 mM $MgCl_2$ and stored as the total membrane fraction. The protein concentration was determined in each fraction by the Bradford assay (Bio-Rad Laboratories), with the protein loading levels normalized to the immunodetection of β-actin and ε-cop.

The contents of Mrp2/MRP2 proteins were determined by immunoblotting using the monoclonal antibodies M2 III-5 for HepG2 and K13 for Hepa 1–6 as described previously [27]. The content of GCLC protein in the cytosolic fraction was determined by immunodetection of the protein using a polyclonal antibody against the human GCLC enzyme X.

Nuclear-extract proteins were isolated from Hepa 1–6 and HepG2 cells. Briefly, cells were collected by centrifugation at 800 × *g* for 5 min at 4 °C and then were resuspended in 400 μl of ice-cold buffer (10 mM HEPES (pH 7.8), 10 mM KC1, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 0.5 mM DTT). After 15 min incubation on ice, NP-40 was added to a final concentration of 0.6%, and cells were vortexed and centrifuged for 1 min at 16,000 × *g* The nuclear pellet was extracted with 50 μl of ice-cold buffer [l0 mM HEPES (pH 7.8), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 25% glycerol] for 30 min at 4 °C on a rocking platform, and debris were removed by centrifugation at 16,000 × *g* for 20 min at 4 °C. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories), and Nrf2 was immunodetected using a commercial anti-Nrf2 antibody.

2.5. GSH assay

The concentrations of total GSH, was measured in cell cultures by the enzymatic recycling procedure using 5,5′-dithiobis-(2-nitrobenzoic acid) and glutathione reductase (DTNB-GSSG reductase recycling assay) [27]. Briefly, cells grown to confluency were harvested 24 h after the described drug treatment and then rinsed with PBS, scraped off the plates, and an aliquot of cell lysates was retained for determining the protein content by the Bradford assay. Cell lysates were centrifuged at 12000 × *g* for 3 min, and the cell pellets were deproteinized by the addition of 5% sulfosalicylic acid. The cells were sonicated for 5 min, centrifuged at 12000 × *g* for 3 min, and the total glutathione was then estimated in the supernatant.

2.6. Statistical analysis

The relative contents of mRNAs in Northern blot experiments were determined using a Phosphorlmager system, and Western blot autoradiographs were analyzed by a densitometer (CS-9000, Shimadzu, Kyoto, Japan). The relative content of a specific mRNA or protein was expressed relative to that of controls. All data are expressed as mean ± S.D. values. A nonpaired Student's *t*-test was used to compare means, with differences considered significantly if the *p* value was less than 0.05.

Table 1 – Primer sequences used for RT-PCR

DNA probe	GenBank accession no.	Primer sequences	Amplified size (bp)
Mouse Mrp2	AF227274	Fwd: 5′-GCTTAGTTCAAGTCTATGGAGT-3′; Rev: 5′-TCCGGCCGATACCGCACTTGATA-3′	822
Human MRP2	E15807	Fwd: 5′-AGCCTGAAGGAAGACGAAGAACTAG-3′; Rev: 5′-CCAATGTCACAAGTGATCCCTC-3′	1163
Mouse GCLC human GCLC	U85414 M90656	Fwd: 5′-GGAGAGGAGAAAAGGTTGTCAT-3′; Rev: 5′-CTTCCCATTGATGATGGTGTCT-3′	995
Mouse GAPDH human GAPDH	M32599 M33197	Fwd: 5′-CTTCATTGACCTCAACTACATGGT-3′; Rev: 5′-TTCACCACCTTCTTGATGTCATC-3′	680

3. Results

3.1. Co-induction ofMrp2/MRP2 and GCLC genes by bucillamine or SA 981

The effects of bucillamine and SA 981 on the expressions of the Mrp2/MRP2 and GCLC genes were assessed in both HepG2 and Hepa 1–6 hepatoma cell lines. Fig. 2Ashows representative Northern blots of the mRNA levels for the MRP2 and GCLC genes in HepG2 cells after 24 h of treatment with either bucillamine or SA 981 at 0.2, 0.5, and 1 mg/ml. Two mRNA transcripts were observed for either MRP2 or GCLC in this human hepatoma cell line. The relative inductions of specific mRNAs of the Northern blots are shown in Fig. 2B. Dose-dependent increases in MRP2 and GCLC mRNA levels were observed after incubation with either bucillamine or SA 981. In the presence of 0.2, 0.5, and 1.0 mg/ml bucillamine, MRP2 mRNA levels increased 1.28 ± 0.05-, 1.42 ± 0.01-, and 1.88 ± 0.02-fold, respectively, and GCLC mRNA levels increased 1.54 ± 0.15-, 2.25 ± 0.10-, and 2.19 ± 0.30-fold, respectively. In the presence of 0.2, 0.5, and 1.0 mg/ml SA 981, MRP2 mRNA levels increased 1.74 ± 0.06-, 2.76 ± 0.57-, and 2.42 ± 0.37-fold, respectively, and the GCLC mRNA levels increased 2.49 ± 0.19-, 4.31 ± 0.66-, and 4.46 ± 0.33-fold, respectively. These experiments indicate that both bucillamine and SA 981 can significantly induce both genes in a dose-dependent manner, with SA 981 appearing to be a stronger inducer than bucillamine in HepG2 cells. In addition, the time course of induction was assessed by Northern blotting with both compounds. The induction of MRP2 and GCLC genes was detected as early as 6 h after incubation with both compounds, with the level of induction peaking at 24 h (data not shown).

As in HepG2 cells, in the murine Hepa 1–6 hepatoma cell line both bucillamine and SA 981 produced significant dose-dependent increases in the mRNA levels of Mrp2 and GCLC after 24 h of exposure, as shown in the representative Northern blot in Fig. 3A. Two transcripts for the Mrp2 gene and a single mRNA transcript for the GCLC gene were identified in Hepa 1–6 cells treated with bucillamine or SA 981. Northern blots showed that in the presence of 0.2, 0.5, and 1.0 mg/ml bucillamine, Mrp2 mRNA increased 2.08 ± 0.13-, 2.62 ± 0.01-, and 3.89 ± 0.36-fold, respectively (Fig. 3B). It is interesting that the induction of Mrp2 mRNA was increased as much as four-fold at the highest dose used. GCLC mRNA levels also increased significantly in the presence of 0.2, 0.5, and 1.0 mg/ml bucillamine, by 1.76 ± 0.1-, 1.89 ± 0.01-, and 2.67 ± 0.24-fold, respectively (Fig. 2B). SA 981 at 0.2, 0.5, and 1.0 mg/ml also increased the mRNA levels of both Mrp2 and GCLC genes: by 1.68 ± 0.04-, 1.97 ± 0.08-, and

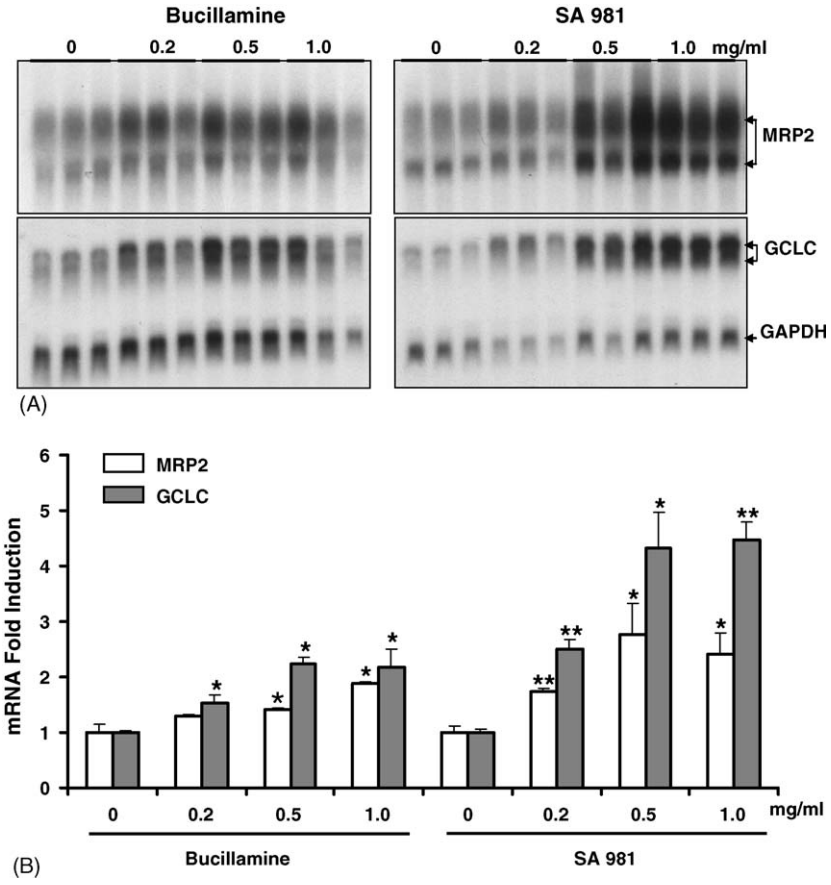

Fig. 2 – Co-induction of MRP2 and GCLC genes by bucillamine and SA 981 in the HepG2 cell line. Total RNA was isolated from control and bucillamine- and SA-981-treated cells. MRP2 and GCLC mRNA levels were analyzed by Northern blotting (A) as described in Section 2. RNA loading was assessed based on hybridization to the GAPDH probe. The relative contents of MRP2 and GCLC mRNAs were determined using a Phosphorlmager system, and are expressed as the induction relative to control values (B). Data are mean ± S.D. values of at least three experiments; $^{*}P < 0.05$; $^{**}P < 0.005$ (Student's *t*-test).

2.43 ± 0.06-fold for Mrp2, and by 1.88 ± 0.06-, 2.33 ± 0.06-, and 2.78 ± 0.04-fold for GCLC, respectively (Fig. 3B).

The similar pattern of co-induction of Mrp2/MRP2 and GCLC genes by bucillamine and SA 981 in human and murine cell lines suggests that the two genes share a conserved molecular mechanism of transcriptional activation in response to exposure to these compounds.

3.2. *Bucillamine and SA 981 induce nuclear Nrf2 translocation in hepatoma cells*

To define the molecular mechanism of Mrp2/MRP2 and GCLC gene induction mediated by bucillamine and SA 981 in both cell lines, the nuclear expression of transcription factor Nrf2 over time was studied by Western blotting. As shown in Fig. 4, both compounds produced a time-dependent increase of Nrf2 protein expression in the nuclear extracts. The levels of Nrf2 protein in the nuclei were maximal at 3 h, remained elevated at 6 h, and decreased to control values at 24 h in both cell lines. Interestingly, SA 981 appeared to be a stronger activator of Nrf2 than did bucillamine. These results suggest that the bucillamine- and SA-981-mediated induction of Mrp2/MRP2 and GCLC genes is due to transcriptional activation of both genes after nuclear translocation of the transcription factor Nrf2 in murine and human hepatoma cells.

3.3. *Bucillamine and SA 981 increase the expressions of Mrp2/MRP2 and GCLC proteins in hepatoma cells*

To determine whether increased contents of Mrp2/MRP2 and GCLC mRNAs were related to increases in the expression of their encoded products, Western blots were performed using proteins from total membrane and cytosolic fractions of HepG2 and Hepa 1–6 cells. Exposure of HepG2 cells to 1 mg/ml bucillamine and SA 981 for 24 h increased the expression of MRP2 and GCLC proteins (Fig. 5A): MRP2 by 2.17 ± 0.17 and 4.22 ± 0.4-fold, respectively, and GCLC by 1.4 ± 0.36 and 4.88 ± 0.85-fold, respectively. However, the increase was only significant for SA 981, as shown in Fig. 5A.

The compounds studied also increased the protein levels of Mrp2 and GCLC in Hepa 1–6 cells, as shown in Fig. 5B. Mrp2 expression increased by 2.23 ± 0.20 and 2.85 ± 0.18-fold after exposure to bucillamine and SA 981 at 1 mg/ml for 24 h, respectively, and GCLC expression increased by 2.04 ± 0.35 and 2.88 ± 0.42-fold, respectively (Fig. 5B).

3.4. *Bucillamine and SA 981 increase GSH biosynthesis in hepatoma cells*

Table 2 lists the changes in GSH content in both cell lines after incubation with 1 mg/ml bucillamine and SA 981 for 24 h. In

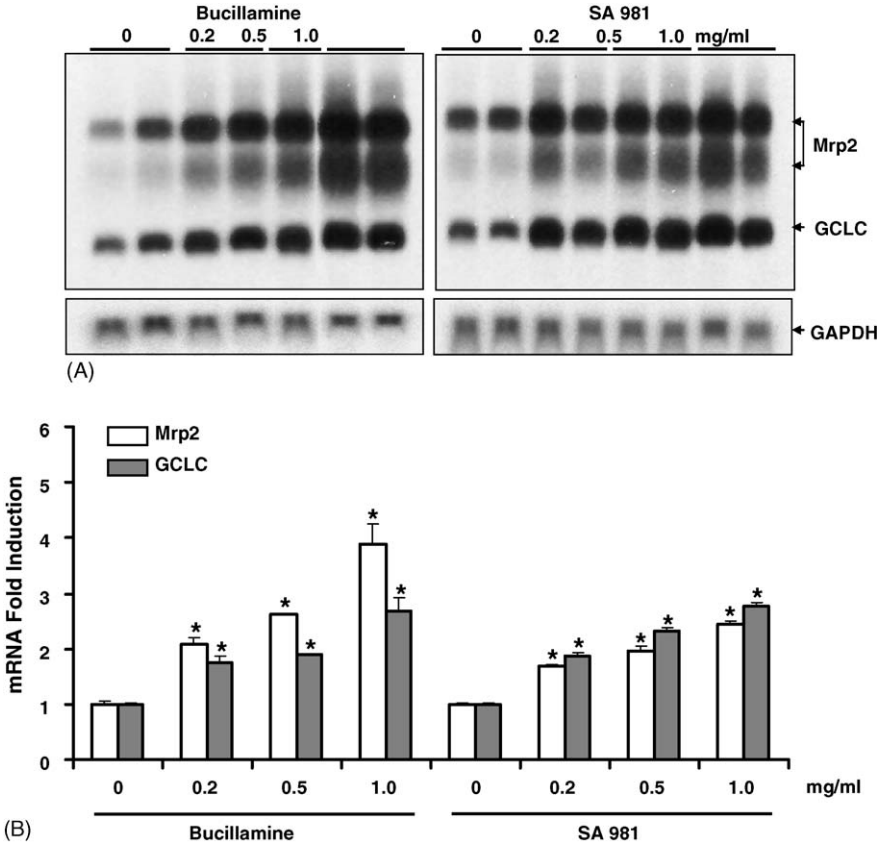

Fig. 3 – Co-induction of Mrp2 and GCLC genes by bucillamine and SA 981 in the Hepa 1–6 cell line. Total RNA was isolated from control and bucillamine- and SA-981-treated cells. Mrp2 and GCLC mRNA levels were analyzed by Northern blotting (A) as described in Section 2. RNA loading was assessed based on hybridization to the GAPDH probe. The relative contents of Mrp2 and GCLC mRNAs were determined using a Phosphorlmager system, and are expressed as the induction relative to control values (B). Data are mean ± S.D. values of at least three experiments; $^{*}P < 0.05$; $^{**}P < 0.005$ (Student's *t*-test).

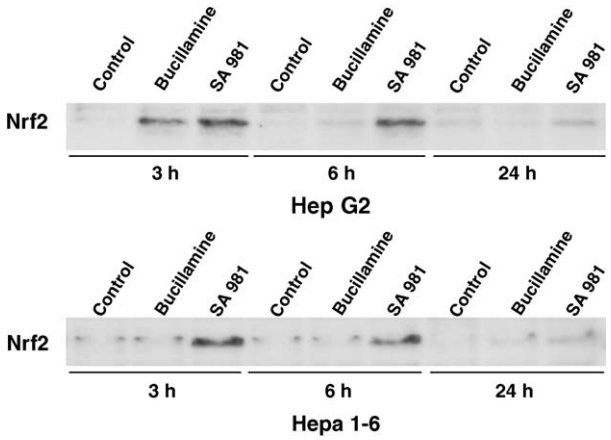

Fig. 4 – Bucillamine and SA 981 induce nuclear Nrf2 translocation in hepatoma cells. Nuclear extract proteins were isolated from control Hepa 1–6 and HepG2 cells, and after 3, 6, and 24 h of incubation with 1 mg/ml bucillamine or SA 981. Nuclear proteins (10 μg) were electrophoresed through an SDS-PAGE gel and transferred onto a nitrocellulose filter. Human and mouse Nrf2 protein was immunodetected using a monoclonal antibody (sc-722X). Consistency between the loading of proteins from different samples was verified by Coomassie blue staining of polyacrylamide gels and Ponceau S staining of the nitrocellulose filter. Two independent experiments were performed.

both cell lines, bucillamine and SA 981 increased the GSH content by two- to three-fold. These results are consistent with the increased levels of GCLC protein in treated cells, which is the rate-limiting enzyme of GSH synthesis.

4. Discussion

Three novel observations were made in the present study: (1) bucillamine and its oxidized metabolite SA 981 increase GSH biosynthesis in both human and murine hepatoma cell lines, suggesting that the antioxidant property of these compounds cannot be attributed only to the donation of thiol groups to glutathione, (2) bucillamine and SA 981 activate the ARE-Nrf2-pathway, increasing the nuclear translocation of this transcription factor, and (3) Nrf2 appears to mediate the coordinated regulation of glutathione biosynthesis and phase III (Mrp2/MRP2) drug metabolism, supporting the interdependence of glutathione-dependent cytotoxic defense systems.

Table 2 – Effect of bucillamine and SA 981 on GSH content in HepG2 and Hepa 1–6 cells

	HepG2 (nmol/mg protein)	Hepa 1–6 (nmol/mg protein)
Control	21.49 ± 0.18	26.23 ± 2.34
Bucillamine	34.45 ± 4.25*	68.97 ± 2.51*
SA981	53.70 ± 6.85*	66.73 ± 7.05*

Data are mean ± S.D. values ($n = 4$); *: $p < 0.05$ relative to control (Student's t-test).

The cytoprotective and anti-inflammatory effects of bucillamine have been demonstrated in several recent models [28–30]. Bucillamine protects myocardial cells *in vitro* from oxidative injury induced by H_2O_2 in a dose-dependent manner [28]. This observation was extended to an *in vivo* model of ischemia/reperfusion, using dogs exposed to coronary artery occlusion and with bucillamine infused at the beginning of reperfusion. The antioxidant markedly reduced the size of myocardial infarcts in a dose-dependent manner [28]. More recently, Amersi et al. demonstrated that bucillamine significantly enhanced the survival and protected against hepatic injury in rats that received liver transplants [29]. Possible mechanisms of this protection include the prevention of excessive accumulation of toxic oxygen species, interruption of redox signaling in hepatocytes, and inhibition of macrophage activation [29]. In the *ex vivo* cold ischemia model of the normal liver, the level of GSH was found to be eight-fold higher in bucillamine-treated livers than in untreated or placebo-treated livers. In addition, a three- to four-fold increase in bile flow in bucillamine-treated livers was observed in this model [29]. Although these findings have been attributed to the antioxidant activity of bucillamine, due to the reduction of GSSG by donated thiol groups preserving a high concentration of glutathione *in vivo* [2,29], the expression of GCLC enzyme and GSH biosynthesis, as well as the expression of the canalicular transporter of GSH into bile (i.e., Mrp2) were not studied in the model. We have previously shown that classical activators of Nrf2-such as the phenolic antioxidant butylated hydroxyanisole induces not only Phase II detoxifying enzymes (Gstal/2) and the rate-limiting enzyme of GSH synthesis (GCLC) in the mouse liver but also the Mrp2 transporter via transcriptional activation of the specific genes [24]. GSH biosynthesis in the liver and GSH output in bile were significantly increased in this model, which produced an increase in bile flow that was independent of the level of bile salts [24]. Further studies are necessary to define the effect of bucillamine and SA 981 on the activation of the ARE-Nrf2 pathway and ARE-driven gene expression in the liver and other tissues in *in vivo* models.

In both of the cell lines investigated in the present study we observed a clear correlation between the levels of GCLC mRNAs, its encoded protein, and GSH content, in both compounds studied. These biological effects of bucillamine and SA 981 are consistent with some antioxidants inducing ARE-driven gene expression, because they are metabolized to pro-oxidants or redox-cycling agents [31]. An additional interesting observation is that the patterns of Mrp2/MRP2 and GCLC gene induction were similar in the murine and human hepatoma cell lines after exposure to bucillamine or SA 981, suggesting that the mechanism of co-induction of these ARE-driven genes in response to exposure to xenobiotics is conserved between species, as previously proposed [24].

Our study demonstrates that bucillamine and SA 981 activate the ARE-Nrf2 pathway, increasing the expression of the ARE-driven genes of GCLC and Mrp2/MRP2. There are several lines of evidence that the activation of the Nrf2 signaling pathway increases the expression of genes that directly detoxify exogenous toxins/carcinogens or endogenous

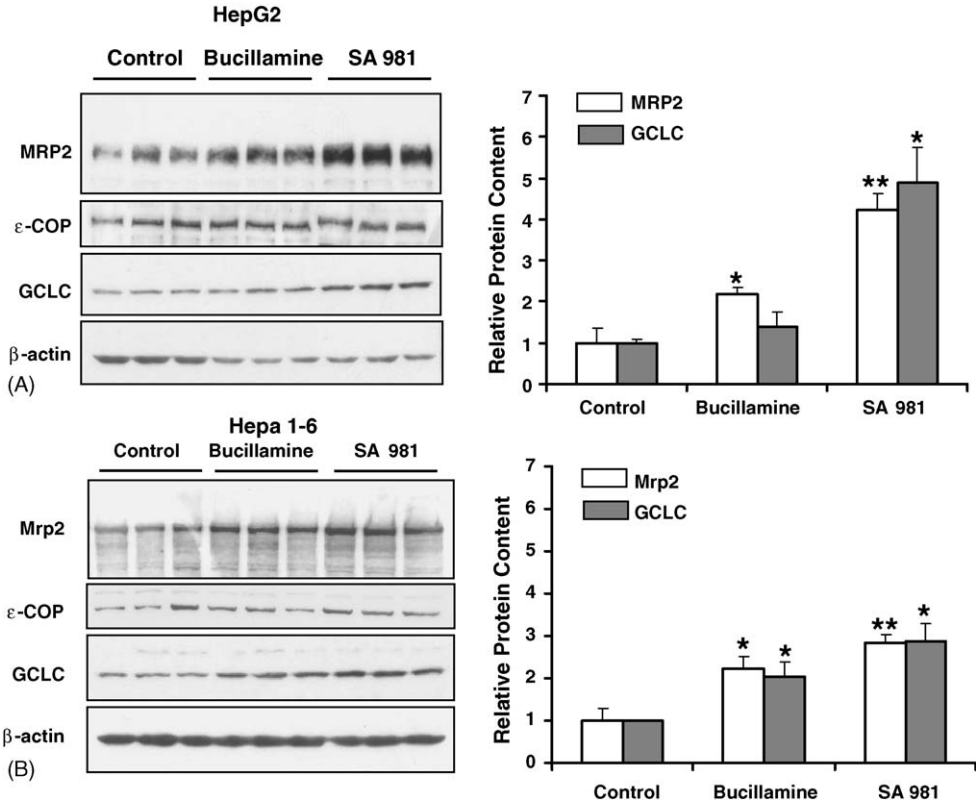

Fig. 5 – Bucillamine and SA 981 increase the expressions of Mrp2/MRP2 and GCLC proteins in hepatoma cells. Proteins were isolated from control HepG2 (A) and Hepa 1–6 (B) cells, and after 24 h of incubation with 1 mg/ml bucillamine or SA 981. Proteins (10 μg) from control and treated cells were separated by SDS-PAGE and transferred onto a nitrocellulose filter. The contents of Mrp2/MRP2 proteins were determined by immunoblotting using the monoclonal antibodies M2 III-5 for HepG2 and K13 for Hepa 1–6. The GCLC protein content was determined by immunodetection of the protein using a polyclonal antibody against the human GCLC enzyme. β-actin and s-cop immunodetection was used as a loading control. The relative contents of MRP2 and GCLC mRNAs were determined by densitometric analysis of the autoradiographs, and are expressed as the induction relative to control values. Data are mean ± S.D. values of at least three experiments; $^{*}P < 0.05$; $^{**}p < 0.005$(Student's *t*-test).

ROS; and also genes involved in the recognition and repair/removal of damaged proteins, which could provide secondary protection against DNA or protein damage that would enhance cell survival [12–32,33]. Thus, the ARE-Nrf2 pathway plays a pivotal role in the regulation of the cellular defense system and is strongly linked to the cytoprotective effects of chemopreventive agents, most of which are dietary components [34–37]. Further studies are necessary to define the potential role of bucillamine as a chemopreventive agent against cancer.

Acknowledgments

We thank Dr. R.P. Oude Elferink and K. Groen (Academic Medical Centre, Amsterdam, The Netherlands) for providing the Mrp2/cM0AT monoclonal antibody M2 III-5, and thank Dr. Lesley McLellan (Biomedical Research Centre, University of Dundee, UK) for providing the human GCLC polyclonal antibody. This work was supported by grant No. 1990510 from the Fondo Nacional de Ciencia y Tecnologia (Fondecyt), Santiago, Chile.

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