High levels of boron promote anchorage-independent growth of nontumorigenic cells.

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Abstract

WHO has presented a health-based guideline value for boron in drinking water. That fact indicates that a high level of boron is toxic for humans. However, there is no direct evidence of boron-mediated malignant transformation. In this study, human lung epithelial nontumorigenic BEAS-2B cells and tumorigenic A549 cells were used to investigate the tumorigenic toxicity of boron *in vitro*. Anchorage-independent growth, a hallmark of malignant transformation, was increased by boron at concentrations of 50, 250 and 500 μM in BEAS-2B cells, though the same concentrations of boron had no influence on anchorage-independent growth of A549 cells. Moreover, boron at concentrations of 250 and 500 μM activated the c-SRC/PI3K/AKT pathway of BEAS-2B cells. The results of our *in vitro* study suggest that exposure to high levels of boron promotes transforming activity of nontumorigenic cells.

Key words: boron, well drinking water, tumorigenic risk, tumorigenic pathway

Abbreviations: inductively coupled plasma-mass spectrophotometer, ICP-MS; mitogen-activated protein kinase kinase, MEK; extracellular signal-regulated kinase, ERK; phosphoinositide 3-kinase, PI3K

1. Introduction

Polluted well drinking water is emerging as an issue of public health in developing countries (Li et al., 2018; Yajima et al., 2017, 2015). Generally, it is difficult to remediate inorganic matter represented by elements in drinking water, while organic matter represented by pathogens can be easily removed by boiling. Therefore, contamination of well drinking water with toxic elements remains a prickly issue in developing countries.

52 Identification of toxic elements in well drinking water could be an initial step to prevent
53 health problems caused by the toxic elements. Previous studies showed contamination of
54 well drinking water with various toxic elements such as arsenic, iron and barium in Asian
55 countries (He et al., 2019; Ilmiawati et al., 2016; Kato et al., 2013; Yajima et al., 2012).
56 Other previous studies showed high levels of boron in drinking water in Argentina and
57 Chile (Table 1). In this study, we newly demonstrated high levels of boron in well drinking
58 water though environmental monitoring in Kabul, Afghanistan (Table 1). Residents in the
59 areas may be exposed to high levels of boron from drinking water.

60 Boron, a ubiquitous element in nature, has a health-based guideline value (2,400
61 μg/L) for drinking water quality proposed by the World Health Organization (WHO),
62 indicating that boron in drinking water is harmful for human health (WHO, 2017). In fact,
63 boron has been reported to have various toxicities including reproductive and
64 developmental toxicities (Khaliq et al., 2018). In contrast, various beneficial effects of
65 boron have been shown in animal and human studies (Nielsen, 2014; Gorustovich et al.,
66 2008; Barranco et al., 2007; Korkmaz et al., 2007). Previous studies also showed that
67 boron could exerted toxicities at quite a high dose (30 g of boric acid) in humans exposed
68 to boron for a short time (ATSDR, 2010). Thus, the toxicities of boron remain controversial.

69 Previous studies have shown that promotion of anchorage-independent growth of
70 nontumorigenic cells is a representative characteristic of malignant transformation that
71 exhibits a change from nontumorigenic cells (non-malignant cells) to tumorigenic cells
72 (malignant cells) (El Khoury et al., 2010; Kato et al., 2002; Kawamoto et al., 2004). Further
73 promotion of anchorage-independent growth of tumorigenic cells is a representative
74 characteristic of progression that exhibits increased malignancy in tumorigenic cells (Kato
75 et al., 2020; Kumasaka et al., 2013). The malignant transformation of nontumorigenic cells
76 and progression of tumorigenic cells are biologically different stages in the process of
77 carcinogenesis (Kato et al., 2020; Omata et al., 2018; Yoshinaga et al., 2018). Activities of

78 c-SRC kinase, phosphoinositide 3-kinase (PI3K) and AKT (PI3K/AKT) and activities of
79 mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase
80 (ERK) (MEK/ERK), potentially sited downstream of c-SRC, are also hallmarks for
81 malignant transformation and progression (Akhand et al., 1999; Kato et al., 2002; Pu et al.,
82 1996; Thang et al., 2015b). Therefore, the combination of the level of anchorage-
83 independent growth and activity of a tumorigenic pathway(s) could be a strong tool for
84 assessing the tumorigenic toxicities of elements *in vitro* (Kumasaka et al., 2013;
85 Yoshinaga et al., 2018). However, there has been no study on the effects of boron on
86 tumorigenic pathways as well as anchorage-independent growth in nontumorigenic cells.
87 In this study, cellular physiological and biochemical studies were performed using
88 cultured normal lung epithelial cells (nontumorigenic BEAS-2B cells) and lung epithelial
89 carcinoma cells (tumorigenic A549 cells) to assess the influences of boron on malignant
90 transformation. Our approach provided a new insight into the health risk of boron
91 exposure.
92
93 **2. Materials and Methods**
94 **2.1. Collection of samples and boron measurements**
95 Sampling of well drinking water (n=227) was performed at ten districts in Kabul,
96 Afghanistan. The methods used for water sampling and elemental analysis were described
97 in detail in our previous report (Kato et al., 2016). Concentrations of 10 toxic elements
98 other than boron in well drinking well water samples were reported in our previous paper
99 (Kato et al., 2016). Boron levels used in this study were previously measured. In brief, all
100 well water was sampled using polyethylene bottles that were rinsed with well water before
101 sample collection. The collected well water samples were then transferred to our
102 department in Nagoya University, Japan and measurements were conducted by an Agilent
103 7700x inductively coupled plasma-mass spectrometry (ICP-MS). As in our previous paper

104 (Kato et al., 2016), approval for this was granted by the Ethical Committees in Nagoya
105 University (no. 2013-0070) and Chubu University (no. 250007 and 20190077) in Japan
106 and the Ministry of Public Health, Islamic Republic of Afghanistan.

107 **2.2. Cell culture**

108 Human nontumorigenic lung epithelial BEAS-2B cells (JCRB, Japan), human lung
109 epithelial carcinoma A549 cells (RIKEN, Japan) (Ohgami et al., 2015) and human
110 nontumorigenic HaCaT keratinocytes (Boukamp et al., 1988) were cultured as described
111 previously (Yajima et al., 2017). BEAS-2B cells and A549 and HaCaT cells were cultured
112 in RPMI-1640 medium (WAKO, Japan) and in DMEM medium (WAKO) containing 10%
113 FBS (Hyclone) and 1% antibiotics complex (WAKO), respectively. Boric acid (H_3BO_3)
114 dissolved in the culture medium was used for sole exposure to boron in our *in vitro*
115 experiments because previous studies showed that >90% of boron and 98.4% of boron
116 are present as boric acid in water in nature (Zeebe et al., 2001) and in physiological fluids
117 in humans (Woods, 1994), respectively. The range of pH values in the culture media in
118 which 5 μM-50,000 μM boric acid was dissolved was 7.12 to 7.18, indicating that pH of the
119 culture media had limited effects in our *in vitro* experiments.

120 **2.3. Anchorage-dependent growth**

121 Anchorage-dependent growth was assessed by crystal violet staining following our
122 previous protocol (Goto et al., 2016). After 24 h of starvation, cells were incubated in the
123 absence or presence of boric acid (WAKO). At 0, 2, 4 and 6 days after treatment with
124 indicated concentrations of boron, cells were fixed using 10% formalin, washed with PBS,
125 dried, and stained by 0.1% crystal violet (CV, Nacalai Tesque, Japan). The CV was
126 extracted with 0.2 M citric acid solution (WAKO). The values of absorbance were detected
127 at 595 nm by a PowerScan4 microplate reader (BioTek, Winooski, VT).

128

129

130 **2.4. Anchorage-independent growth**

131 Anchorage-independent growth was assessed using a colony formation assay following
132 our previously reported method (Yajima et al., 2015). After cells had been pre-treated in
133 the absence or presence of boron for 4 days, 4×10^3 BEAS-2B cells, 1×10^3 A549 cells or
134 2×10^4 HaCaT cells were resuspended in 1 mL RPMI-1640 or DMEM medium containing
135 1% methylcellulose (WAKO). The cells were then incubated in the absence or presence of
136 boron (5, 50, 250, 500 and 5,000 μM) in 24-well ultra-low adhesion plates (Corning, NY).
137 After 14 days of incubation, the colonies (diameter ≥50 μm) were counted. PP2 (EMD
138 Biosciences, CA) was applied to inhibit c-SRC.

139 **2.5. Immunoblot analysis**

140 c-SRC, PI3K/AKT and MEK/ERK are representative tumorigenic factors that regulate
141 malignant transformation (Akhand et al., 1999; Kato et al., 2020; Thang et al., 2015b).
142 These oncogenic signaling molecules are activated by the phosphorylation of their critical
143 tyrosine (Tyr), serine (Ser) and/or threonine (Thr) (Kato et al., 2002; Thang et al., 2015a).
144 In order to evaluate boron-mediated activities of the oncogenic signaling molecules,
145 immunoblotting was conducted following our previously reported protocols (Kato et al.,
146 2004). Primary antibodies to phospho-c-SRC (Tyr416), c-SRC, phospho-PI3K p55
147 (Tyr199), PI3K, phospho-AKT (Ser473), AKT, phospho-MEK1/2 (Ser217/221), MEK1/2,
148 phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 were purchased from Cell Signaling
149 (Danvers, MA). Primary antibodies to PI3K p55 and α-TUBULIN were obtained from Santa
150 Cruz Biotechnologies and Sigma-Aldrich Corporation, respectively. HRP-conjugated
151 secondary antibodies were provided by Calbiochem (EMD Biosciences, CA) and Cell
152 Signaling.

153 **2.6. Statistical analysis**

154 Results are presented as means ± standard deviation (SD) and were analyzed with SPSS
155 25.0 (IBM Corp., Armonk, NY). One-way analysis of variance (ANOVA) with Dunnett's t-

156 test or Bonferroni's post hoc test was used for multiple group comparisons. Two-sided
157 $p<0.05$ was judged as significant.
158
159 **3. Results**
160 **3.1. Levels of boron in well water in Afghanistan**
161 Boron levels in well drinking water in Kabul, Afghanistan are presented in Table 1. The
162 mean boron concentration in well drinking water samples was 2,656 μg/L (246 μM), which
163 exceeds the reference (2,400 μg/L) for drinking water recommended by WHO (WHO,
164 2017). The maximum boron concentration in well drinking water in Kabul was 23,395 μg/L
165 (2,164 μM). Both the mean and maximum boron concentrations in drinking water collected
166 from Afghanistan were higher than those previously reported for other countries, while the
167 reported median concentration of boron in drinking water in the north of Chile (2,900 μg/L)
168 (Cortes et al., 2011) is higher than that in Kabul (1,619 μg/L) in this study. The reason for
169 the high level of boron in well water in Kabul is unclear despite the fact that maximum
170 levels of chromium (66.0 μg/L), arsenic (104.6 μg/L), cadmium (0.2 μg/L), mercury (0.4
171 μg/L), and lead (4.7 μg/L) in the same water were comparable with the levels in other
172 areas previously reported (Ilmiawati et al., 2016; Kato et al., 2016, 2013, 2010). Since vast
173 natural boron deposits such as deposits of barite, evaporite and pegmatite have been
174 reported in areas surrounding Kabul Basin (Mack et al., 2010; Peters et al., 2007), the
175 deposits could be potential sources of high levels of boron in well water in Kabul.
176 **3.2. Influence of boron on anchorage-dependent growth**
177 Although boron at concentrations of 5-5,000 μM did not suppress anchorage-dependent
178 growth of BEAS-2B cells, boron at a concentration of 50,000 μM significantly suppressed
179 anchorage-dependent growth (Fig. 1A). On the other hand, boron at concentrations of both
180 5,000 μM and 50,000 μM significantly suppressed anchorage-dependent growth of A549
181 cells (Fig. 1B).

182 **3.3. Influence of boron on anchorage-independent growth**

183 Boron at concentrations of 50-500 μM induced anchorage-independent growth of BEAS-
184 2B cells in a concentration-dependent manner (Fig. 2A, B), but the equivalent
185 concentrations of boron had no effect on the growth of A549 cells (Fig. 2C, D). These
186 results indicated different sensitivities of nontumorigenic cells and tumorigenic cells to 50-
187 500 μM boron for anchorage-independent growth. On the other hand, boron at a
188 concentration of 5,000 μM significantly suppressed anchorage-independent growth of
189 BEAS-2B and A549 cells (Fig. 2A-D). Our results suggest an anti-cancer effect of boron at
190 a concentration of 5,000 μM, which seems to be an unphysiologically high level based on
191 previous studies (Moseman, 1994).

192 **3.4. Boron-mediated activation of c-SRC and PI3K/AKT pathways**

193 Based on our results for boron-mediated anchorage-independent growth (Fig. 2), the
194 effects of boron on activities of tumorigenic factors (c-SRC, PI3K/AKT and MEK/ERK),
195 which have been reported to regulate anchorage-independent growth (Kato et al., 2020;
196 Thang et al., 2015b), in nontumorigenic BEAS-2B cells and tumorigenic A549 cells were
197 investigated. Expression and phosphorylation levels of c-SRC, PI3K/AKT and MEK/ERK
198 molecules in BEAS-2B cells cultured under an anchorage-independent condition in the
199 presence or absence of boron (250 and 500 μM) are shown in Figure 3. Phosphorylation
200 levels of c-SRC and PI3K/AKT were increased in BEAS-2B cells treated with boron.
201 However, there were very limited effects of boron on phosphorylation levels of MEK/ERK.
202 These results indicate that boron activates c-SRC and PI3K/AKT tumorigenic pathways
203 but not the MEK/ERK tumorigenic pathway (Figure 3, left). On the other hand, boron had
204 limited influence on phosphorylation levels (activities) of the tumorigenic factors in A549
205 cells (Figure 3, right).

206

207 **3.5. Influence of c-SRC on boron-mediated increase in anchorage-independent**
208 **growth**
209 In order to confirm the influence of boron-mediated c-SRC activation on anchorage-
210 independent growth of BEAS-2B cells, further study using PP2, a c-SRC kinase inhibitor
211 (Thang et al., 2011), was performed. After decreased c-SRC activity in BEAS-2B cells
212 treated with PP2 was confirmed (Figure 4A), the influence of c-SRC activity on boron-
213 mediated anchorage-independent growth of BEAS-2B cells was examined. The
214 pharmacological inhibition of c-SRC significantly suppressed anchorage-independent
215 growth of the cells promoted by 500 μM boron (Figure 4B, C), suggesting that c-SRC is
216 one of the crucial molecules for regulation of boron-mediated malignant transformation.
217
218 **4. Discussion**
219 In this study, the effects of high levels of boron on two types of cell growth were
220 investigated in human lung epithelial BEAS-2B nontumorigenic cells and human lung
221 epithelial A549 tumorigenic cells *in vitro*. Boron at concentrations of 50-500 μM promoted
222 anchorage-independent growth of not only BEAS-2B cells but also human HaCaT
223 nontumorigenic keratinocytes (Supplemental Figure 1), indicating boron-mediated
224 malignant transformation in nontumorigenic cells. On the other hand, the equivalent
225 concentrations of boron showed limited effects on the growth and tumorigenic signaling of
226 tumorigenic A549 cells, indicating a limited effect of boron on progression in tumorigenic
227 cells.
228 Our biochemical study was then implemented to further characterize the molecular
229 mechanism of boron-mediated modulation of transforming activity in BEAS-2B
230 nontumorigenic cells. Boron (250 and 500 μM) activated the c-SRC and PI3K/AKT
231 pathways but not the MEK/ERK pathway potentially sited downstream of c-SRC in BEAS-
232 2B cells (Thang et al., 2015b, 2011). Based on results of previous study and our studies,

233 c-SRC activation by boron via phosphorylation of Tyr416 in c-SRC seems to be the first
234 step of boron-mediated activation of oncogenic signaling (Akhand et al., 1999; Guarino,
235 2010; Thang et al., 2015b). Activated c-SRC then activates PI3K, which directly binds c-
236 SRC through the SH3 domain, by phosphorylation of Tyr199 in PI3K. Activated PI3K is
237 thought to in turn induce the activation of AKT through phosphorylation of Ser473 of AKT.
238 Our biochemical results for boron-mediated activation of the oncogenic pathway of c-
239 SRC/PI3K/AKT (Kato et al., 2002; Thang et al., 2015b) in BEAS-2B cells again suggest
240 promotion of the malignant transformation of nontumorigenic cells by boron. Our results
241 showing that the boron-mediated increase of anchorage-independent growth (transforming
242 activity) was suppressed by a c-SRC inhibitor in BEAS-2B cells indicate that c-SRC is
243 involved in the boron-mediated promotion of malignant transformation of nontumorigenic
244 cells. The promotion of malignant transformation of nontumorigenic cells found in this
245 study does not conflict with anti-cancer effects of boron on decreased levels of anchorage-
246 dependent growth of transformed tumorigenic cells (carcinoma cells) found in previous
247 studies (Acerbo and Miller, 2009; Barranco et al., 2009; Scorei et al., 2008). In fact, both
248 anti-cancer effects on tumorigenic cells and cancer-promoting effects on nontumorigenic
249 cells are well-known effects of arsenic (Thang et al., 2014; Yajima et al., 2015).
250 Model animals for cancer could be strong tools for evaluating the malignant
251 transformation *in vivo* (Kato et al., 2004; Kumasaka et al., 2010). In fact, malignant
252 transformation of thyroid cells was promoted by oral co-exposure to boron, cadmium and
253 molybdenum in rats in a previous study (Luca et al., 2017). However, there has been no
254 animal study in which the effect of exposure to only boron on malignant transformation
255 was investigated. Moreover, there is no direct evidence for carcinogenic toxicity of boron in
256 epidemiological studies in humans. Polyhedral approaches targeting cells, animals and
257 humans are needed to investigate the tumorigenic toxicity of exposure to boron.
258

259 **Figure legends**

260 **Fig. 1. Influence of boron on anchorage-dependent growth of BEAS-2B cells and**
261 **A549 cells.** Ratios (means ± SD) of cell viability of BEAS-2B cells (n=4) (**A**) and A549 cells
262 (n=3) (**B**) in the absence or presence of boron (5, 50, 500, 5,000 and 50,000 μM) for the
263 indicated days are presented. Significant differences from nil control by ANOVA with
264 Dunnett's t-test (*, $p < 0.05$; **, $p < 0.01$).

265 **Fig. 2. Influence of boron on anchorage-independent growth of BEAS-2B cells and**
266 **A549 cells.** Ratios (means ± SD) of colony numbers (**A**, **C**) and representative
267 photographs (**B**, **D**) of BEAS-2B cells (n=3) (**A**, **B**) and A549 cells (n=4) (**C**, **D**) in the
268 absence or presence of boron (5, 50, 250, 500 and 5,000 μM) are presented. Significant
269 differences from nil control by ANOVA with Dunnett's t-test (*, $p < 0.05$; **, $p < 0.01$). Scale
270 bars:100 μm.

271 **Fig. 3. Influence of boron on activities of carcinogenic molecules.** Expression and
272 phosphorylation levels of c-SRC and pathways of PI3K/AKT and MEK/ERK in
273 nontumorigenic BEAS-2B cells (left) and tumorigenic A549 cells (right) in the absence or
274 presence of boron (250 and 500 μM) are presented. P-c-SRC, phosphorylated c-SRC; P-
275 PI3K, phosphorylated PI3K; P-AKT, phosphorylated AKT; P-MEK, phosphorylated MEK,
276 P-ERK, phosphorylated ERK. α-TUBULIN was used as an internal control.

277 **Fig. 4. Influence of c-SRC activity on promotion of anchorage-independent growth**
278 **by boron.** BEAS-2B cells were cultured under an anchorage-independent condition in the
279 presence or absence of boron (500 μM) and/or PP2 (1 μM). Phosphorylation levels and
280 expression levels of c-SRC and α-TUBULIN in the cells are presented (**A**). Ratios
281 (means ± SD) of colony numbers (**B**) and representative photographs (**C**) of BEAS-2B
282 (n=4) in the presence or absence of boron (500 μM) and PP2 (1 μM) are presented.
283 Significant differences among groups by Bonferroni's post hoc test (*, $p < 0.05$; **, $p <$
284 0.01). Scale bars: 100 μm.

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Competing interests

All authors declare that they have no competing interests.

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Figure 1

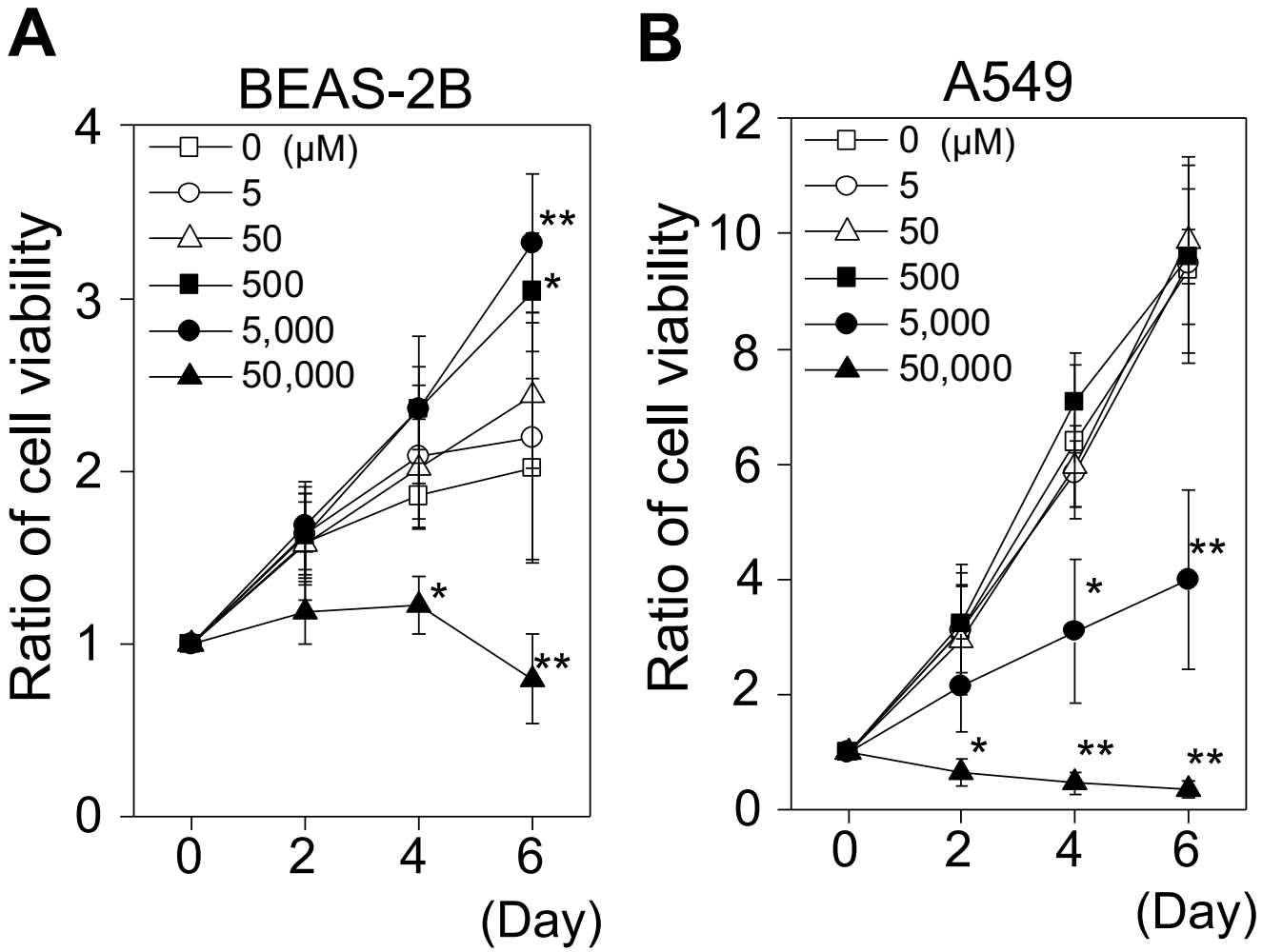
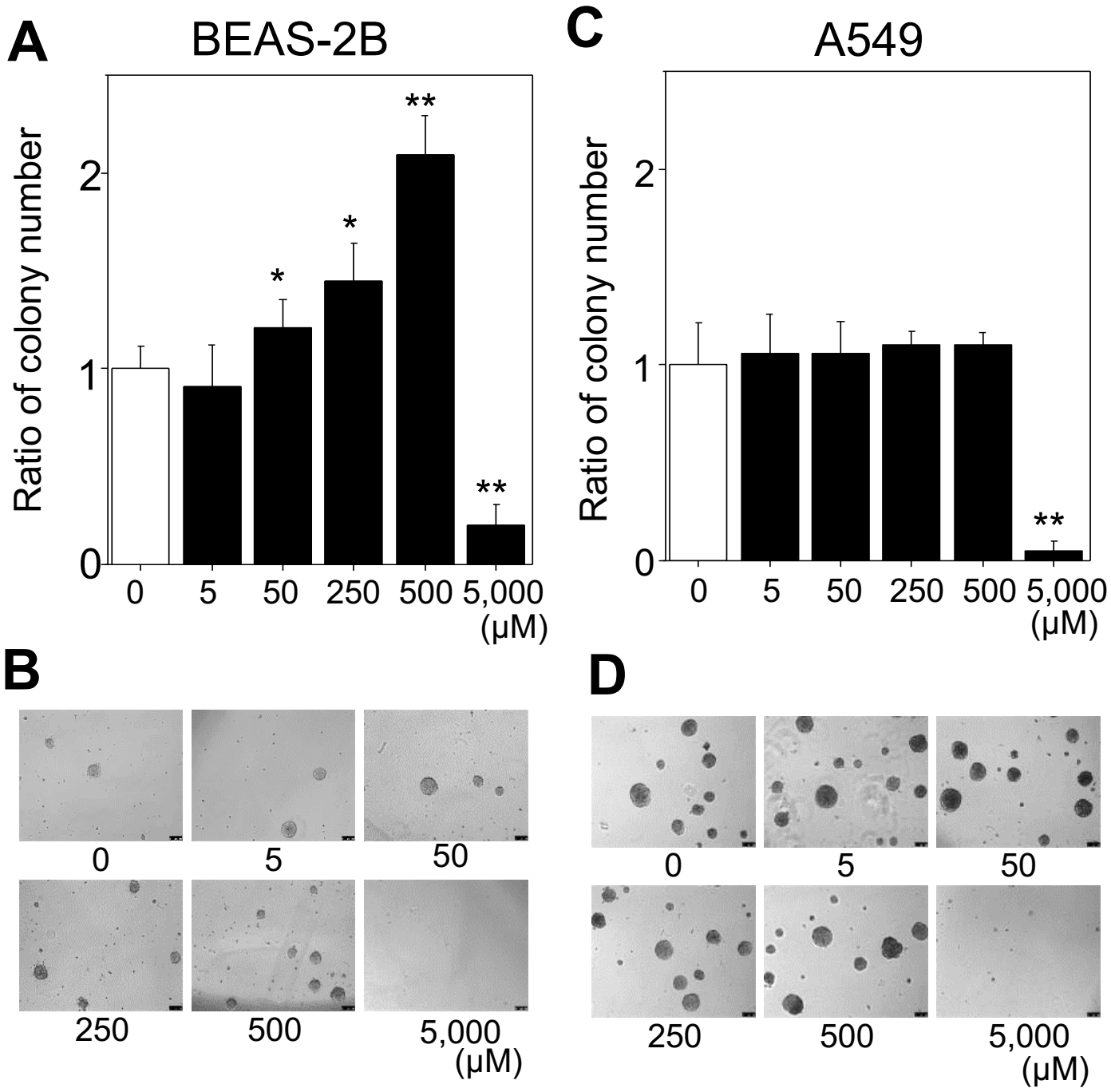

A
BEAS-2B
Ratio of cell viability
0 (μM)
5
50
500
5,000
50,000
(Day)
B
A549
Ratio of cell viability
0 (μM)
5
50
500
5,000
50,000
(Day)

Figure 2


A
BEAS-2B
Ratio of colony number
0
1
2
*
*
**
**
0
5
50
250
500
5,000
(μM)
C
A549
Ratio of colony number
0
1
2
**
0
5
50
250
500
5,000
(μM)
B
0
5
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500
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(μM)
D
0
5
50
250
500
5,000
(μM)

Figure 3

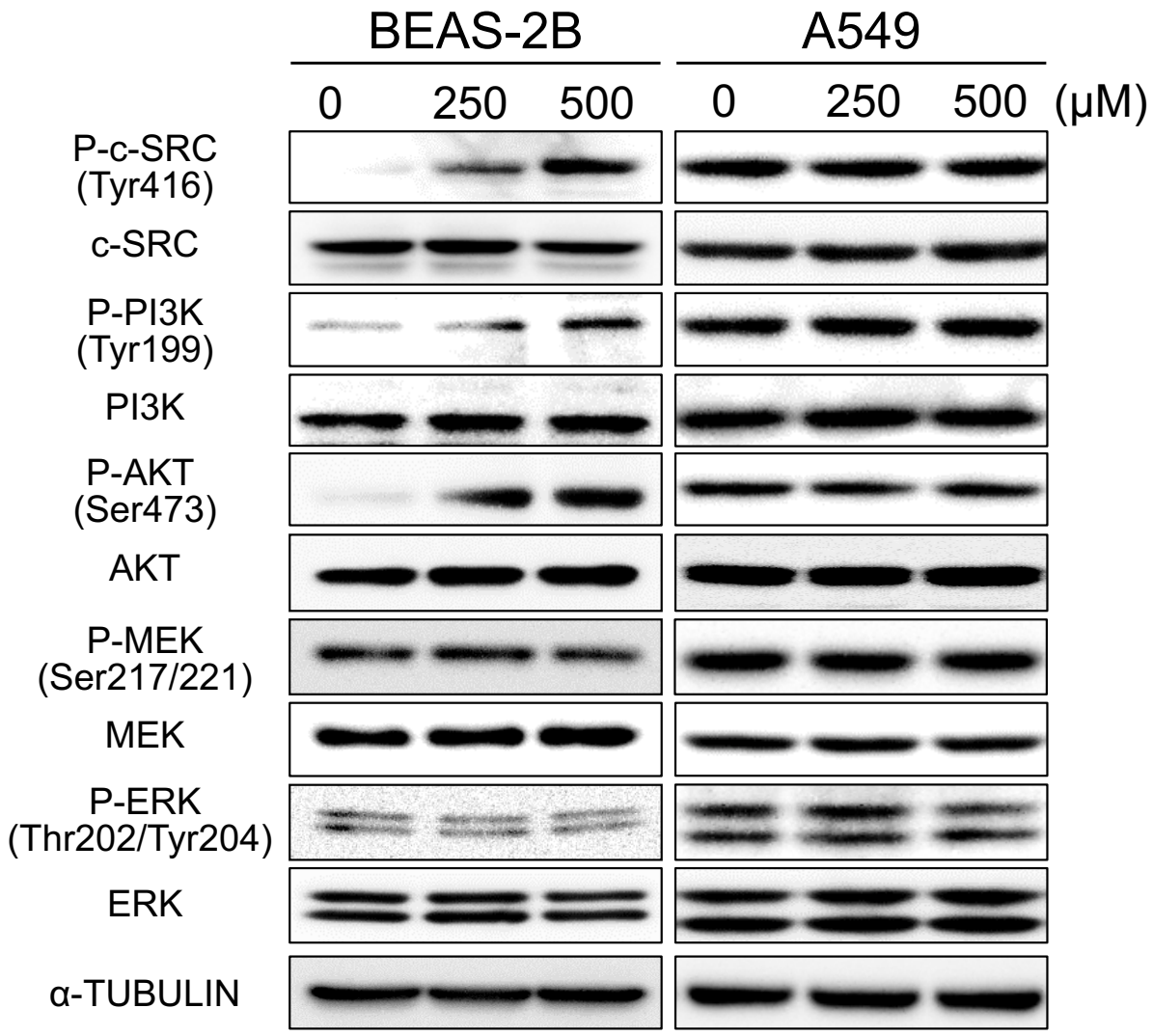
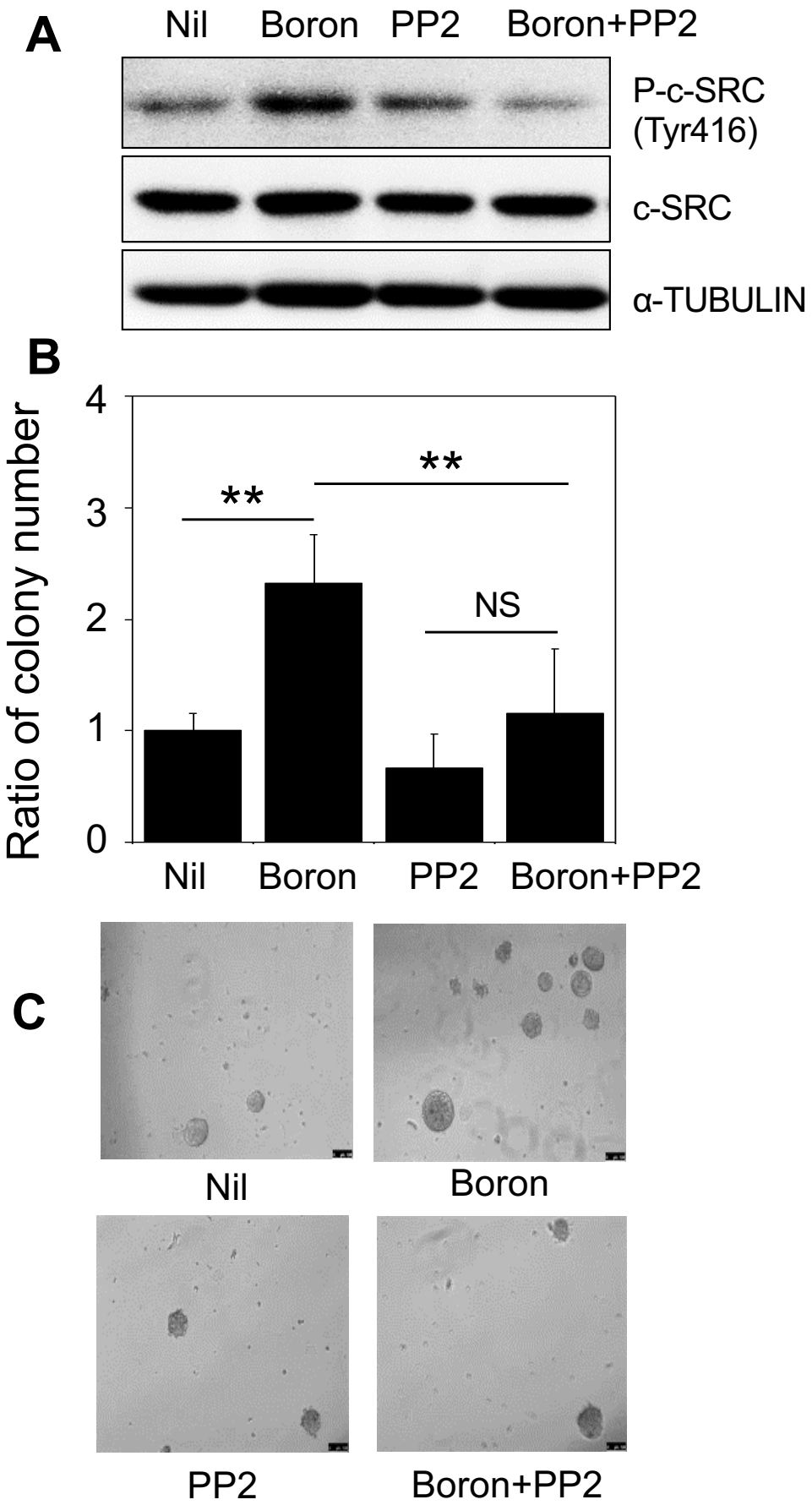

BEAS-2B
A549
0
250
500
0
250
500
(μM)
P-c-SRC
(Tyr416)
c-SRC
P-PI3K
(Tyr199)
PI3K
P-AKT
(Ser473)
AKT
P-MEK
(Ser217/221)
MEK
P-ERK
(Thr202/Tyr204)
ERK
α-TUBULIN

Figure 4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Nil
Boron
PP2
Boron+PP2
P-c-SRC
(Tyr416)
c-SRC
α-TUBULIN
B
Ratio of colony number
4
3
2
1
0
**
**
NS
Nil
Boron
PP2
Boron+PP2
C
Nil
Boron
PP2
Boron+PP2

489 **Table 1. Boron levels measured by ICP-MS in drinking water in the world.**

Country	Sample type	No.	Average (μg/L)	Range (μg/L)	Reference
Afghanistan	**Well water**	**227**	**2,656[a]**	**83~23,395**	**This study**
Sweden	Well water	89	9[b]	0.7~106	(Rosborg et al., 2003)
Italy	Tap water	15	17[b]	0~76	(Cidu et al., 2011)
China	Drinking water	98	46[a]	3~337	(Xu et al., 2010)
Laos	Well water	61	90[b]	5.2~1,997	(Chanpiwat et al., 2011)
Malaysia	Well water	21	96[a]	5.9~195.1	(Kato et al., 2010)
Turkey	Tap water	88	1,700[a]	30~3,390	(Çöl and Çöl, 2003)
Argentina	Drinking Water	10	2,004[a]	335~ 5,956	(Concha et al., 2010)
Chile	Drinking water	173	2,900[b]	220~11,300	(Cortes et al., 2011)

490 [a] Mean, [b] Median.