Genotoxic Evaluation of Surfactin C in Chinese Hamster Lung Cell Line

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To investigate the mutation inducibility of surfactin C, we performed the chromosome aberration assay with Chinese hamster lung cells in vitro. The colorimetric MIT screening assay was carried out to determine the cytotoxicity index (IC50) of surfactin C. The IC50 value was 125 μg/ml. For the chromosome aberration test of surfactin C, the maximum concentration was employed as 125 μg/ml, followed by 62.5 and 31.25 μg/ml for the lower concentrations, with or without metabolic activation (S9). Cyclophosphamide and mitomycin C were used as positive controls in the presence and absence of S9 metabolic activation, respectively. These results showed that surfactin C was not capable of inducing chromosome aberration, as measured by the chromosome aberration test using Chinese hamster lung cell line. There is no evidence for surfactin C to have a genotoxic potential.

Key words: Surfactin C, Chromosome aberration test, MIT test, Chinese Hamster Cell

INTRODUCTION

Biosurfactants are proteins with detergent, emulsifier, and antimicrobial actions that have potential application in environmental applications such as the treatment of organic pollutants and oil recovery (Georgiou et al., 1990; Desai and Banat, 1997; Banat et al., 2000; Rodrigues et al., 2006). Especially, microbial surfactants have several advantages over synthetic surfactants such as lower toxicity, easier biodegradability, better environmental compatibility, higher selectivity and specific activity at extreme temperatures, pH and salinity (Georgiou et al., 1990; Desai and Banat, 1997; Banat et al., 2000; Rodrigues et al., 2006).

Surfactin showed that it had a strong surface tension-lowering activity and showed antiviral, antitumor, fibrinolytic and hypocholesterolemic activities (Arima et al., 1968; Singh and Cameotra, 2004; Mulligan, 2005). It is a mixture of isofoms which slightly differ in their physiological properties due to a variation in the chain length and branching of its hydroxy fatty acid component as well as substitutions of the amino acid components of the peptide ring (Kanatomo et al., 1995). Among of them, surfactin C (Fig. 1) enhanced endogenous thrombolytic reactions by activation of plasminogen activator and inhibition of platelet aggregation (Kikuch and Hasumi, 2002; Lim et al., 2005). It showed antimicrobial activity against methicillin-resis-

Fig. 1. The structure of surfactin C.
tant Staphylococcus aureus (MRSA) and inhibited various inflammatory mediators such as cyclooxygenase-
2, interleukin-1β and inducible nitric oxide synthase (Hwang et al., 2005a, b; Takahashi et al., 2006). Moreover, it was less toxic than other surfactants as judged from the results of an acute toxicity study in mice (Pakr et al., 2006). Hwang et al. (2008) demonstrated that it showed no genetic toxicity in bacterial reverse mutation and mouse micronucleus assay. The chromosome aberration test using cultured mammalian cells is one of the sensitive methods to predict environmental mutagens and/or carcinogens and is a complementary test to the bacterial reverse mutation test. There is no literature about the genetic toxicity of surfactin C in mammalian cell lines. Therefore, we tested its clastogenicity in cultured mammalian cells, Chinese hamster lung (CHL) cells.

**MATERIALS AND METHODS**

**Chemicals.** Surfactin C (purity > 98%) produced from *Bacillus subtilis* BC1212 was kindly obtained from B&C Biopharm (Suwon, Korea). Cyclophosphamide (Sigma, USA) and mitomycin C (Singm, USA) were used as positive controls. Rat liver S9 induced with Aroclor 1254 (Moltox Inc, USA) was used for metabolic activation.

**Chinese hamster lung (CHL) cell line.** CHL cells were purchased from the American Type Culture Collection (ATCC, USA) and cultured in Eagle’s minimal essential medium (EMEM, Hyclone, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), in a 37°C incubator containing 5% CO2 without any antibiotics or anti-mycotics. Subcultures were carried out every 2 days, using 0.03% Puck’s EDTA and 0.25% Trypsin-EDTA solutions.

**MTT assay.** Cytotoxicity assay was performed using a two-fold serial dilution gradient of surfactin C concentrations, ranging from 15.6–1000 μg/mL. All treatments were performed in triplicate and the cells were cultured for 18 h in the presence and absence of surfactin C. An IC50 concentration was determined from each of the three experiments and the final IC50 was determined by averaging the values of the three experiments (± SD). The IC50 is defined as the cytotoxicity index that reduces the cell number (by growth inhibition and/or cell killing) to 50% compared with untreated-control CHL cells. The IC50 value was used to determine the chromosomal aberration assay concentration ranges.

**Chromosomal aberration assay.** The assay was performed according to OECD and scientific guidelines (Galloway et al., 1994; OECD, 1997). For the short-period assay with or without S9 mix, CHL cells (2 × 105 cells/0.5 ml) were seeded in a 24 well plate culture dish, and incubated in a culture medium for overnight. The surfactin C was added with or without S9 mix and the cultures were incubated for 6 h. After the 6 h treatment, the cells were washed with phosphate buffered saline (PBS), and incubated in fresh culture medium for a further 18 h. The continuous treatment test was carried out for 24 h treatment without S9 mix. PBS was used as a negative control, while 20 μg/ml cyclophosphamide (Sigma, USA) and 0.1 μg/ml mitomycin C (Sigma, USA) were used as positive controls for experiments conducted with and without metabolic activation, respectively (Galloway et al., 1994). The cells were treated with colchicine (Sigma, USA; 0.1 μM of final concentration) 2 h before cell harvesting. The cells were trypsinized and incubated in a 75 mM hypotonic KCl solution for 20 min at 37°C. Fixed with acetic acid-ethanol (1:3 by volume), and then spread onto clean glass slides. Each slide was stained with 5% Giemsa solution.

Duplicate cultures were used for each experiment. Cells were harvested 24 h after treatment initiation and a minimum of 200 metaphases (100 from each of two duplicate cultures) were analyzed for chromosome damage. Aberrations were classified according to Scott et al. (1983) into chromosome and chromatid type damage, with further subdivision into deletions and exchanges. Polyploidy and endoreduplication were recorded as a percentage per 100 metaphases counted.

The frequencies of structural or numerical aberrations were evaluated by the following criteria; less than 5%, 5% to less than 10%, and 10% or more were defined as negative (−), equivocal (±) and positive (+), respectively. The result was considered to be positive if reproducibility was confirmed. Total frequencies of structural aberrations excluded the frequencies of aberrant cells that have gaps only without other aberrations.

**Statistical analysis.** Statistical analysis was performed using SPSS 12.0k. The differences in the frequency of chromosomal aberrations between groups treated with surfactin C and controls were analyzed by the Fisher’s exact test. P-values of less than 0.05 were considered to be consistent with statistical significance.

**RESULTS AND DISCUSSION**

Genotoxicity is the study of the toxic effects of chemical or physical substances to the gene pool, where
Table 1. Summary of results obtained from chromosomal aberration test in CHL cells treated with 6 hr short-treatments of surfactin C

| Compound   | Conc. (µg/ml) | S9 mix | Time (hr) | Aberrated cell (%)\(^2\) | -g | +g |
|------------|---------------|--------|-----------|---------------------------|----|----|
| PBS        | 0             | -      | 6/18      | 0                         | 0  | 0  |
| Surfactin C| 125           | -      | 6/18      | 2                         | 3  |    |
|            | 62.5          | -      | 6/18      | 1                         | 2  |    |
|            | 31.25         | -      | 6/18      | 1                         | 1  |    |
| MMC        | 0.1           | -      | 6/18      | 42\(^*\)                  | 44\(^*\) |    |
| PBS        | 0             | +      | 6/18      | 1                         | 1  |    |
| Surfactin C| 62.5          | +      | 6/18      | 2                         | 3  |    |
|            | 31.25         | +      | 6/18      | 2                         | 2  |    |
| CP         | 20            | +      | 6/18      | 54\(^*\)                  | 56\(^*\) |    |

MMC, Mitomycin C; CP, Cyclophosphamide.

\(^1\)Treatment time (exposure time/new medium time).

\(^2\)\% of cells with chromosome aberrations; +g, \% of cells with chromosome aberrations + \% of cells with gaps.

\(^*\)Significantly greater than the corresponding vehicle control, \(p < 0.001\).

Table 2. Metaphase analysis of chromosomal aberration test in CHL cells treated with 6 hr short-treatments of surfactin C

| Compound   | Conc. (µg/ml) | S9 mix | Time (hr) | ctb | cSB | cTE | cSE | Aberration excluding gap (%) | ctg | csg | pol | endo | Aberration including gap (%) |
|------------|---------------|--------|-----------|-----|-----|-----|-----|-------------------------------|-----|-----|-----|------|-------------------------------|
| PBS        | 0             | -      | 6/18      | 0   | 0   | 0   | 0   | 0                             | 0   | 0   | 0   | 0    | 0                             |
| Surfactin C| 125           | -      | 6/18      | 2   | 0   | 0   | 0   | 2                             | 1   | 0   | 0   | 0    | 3                             |
|            | 62.5          | -      | 6/18      | 1   | 0   | 0   | 0   | 1                             | 1   | 0   | 0   | 0    | 2                             |
|            | 31.25         | -      | 6/18      | 1   | 0   | 0   | 0   | 1                             | 0   | 0   | 0   | 0    | 1                             |
| MMC        | 0.1           | -      | 6/18      | 20  | 2   | 18  | 2   | 44                            | 2   | 0   | 0   | 0    | 44                            |
| PBS        | 0             | +      | 6/18      | 1   | 0   | 0   | 0   | 1                             | 0   | 0   | 0   | 0    | 1                             |
| Surfactin C| 125           | +      | 6/18      | 2   | 0   | 0   | 0   | 2                             | 1   | 0   | 0   | 0    | 3                             |
|            | 62.5          | +      | 6/18      | 1   | 0   | 1   | 0   | 2                             | 0   | 0   | 0   | 0    | 2                             |
|            | 31.25         | +      | 6/18      | 1   | 0   | 0   | 0   | 1                             | 1   | 0   | 0   | 0    | 2                             |
| CP         | 20            | +      | 6/18      | 25  | 5   | 22  | 2   | 54                            | 2   | 0   | 0   | 0    | 56                            |

MMC, Mitomycin C; CP, Cyclophosphamide.

\(^1\)Treatment time (exposure time/new medium time).

\(^2\)ctb, chromatid break; cSB, chromosome break; cTE, chromatid exchange; cSE, chromosome exchange; ctg, chromatid gap; csg, chromosome gap; pol, polyploidy; endo, endoreduplication.
Table 4. Metaphase analysis of chromosomal aberration test in CHL cells treated with 24 hr short-treatments of surfactin C

| Compound | Conc. (µg/ml) | S9 mix | Time (hr) | No. of structural aberration (%)* |
|----------|--------------|--------|-----------|----------------------------------|
|          |              |        |           | ctb | csb | cte | cse | Aberration excluding gap (%) | ctb | csb | pol | endo | Aberration including gap (%) |
| PBS      | 0            | -      | 24        | 1   | 0   | 0   | 0   | 1                              | 0   | 0   | 0   | 0   | 1                              |
| Surfactin C | 125        | -      | 24        | 2   | 0   | 0   | 0   | 2                              | 1   | 0   | 0   | 0   | 3                              |
|          | 62.5        | -      | 24        | 2   | 0   | 0   | 0   | 2                              | 1   | 0   | 0   | 0   | 3                              |
|          | 31.25       | -      | 24        | 1   | 1   | 1   | 0   | 2                              | 0   | 0   | 0   | 0   | 2                              |
| MMC      | 0.1         | -      | 24        | 21  | 3   | 18  | 1   | 43                            | 2   | 0   | 0   | 0   | 45                             |

MMC, Mitomycin C; CP, Cyclophosphamide.
*Treatment time (exposure time).
**ctb, chromatid break; csb, chromosome break; cte, chromatid exchange; cse, chromosome exchange; ctg, chromatid gap; csg, chromosome gap; pol, polyplody; endo, endoreduplication.

the 24 hr (Table 3 and 4). Based on these data, surfactin C did not induce structural chromosomal aberrations in the short-treatments and continuous treatment. The results suggested that surfactin C has no clastogenic potential in cultured mammalian cells either with or without S9 activation. This is consistent with the previous reports that surfactin C is negative with or without metabolic activation in mutation assay using Salmonella typhimurium and Escherichia coli (Hwang et al., 2008).

REFERENCES

Arima, K., Kakinuma, A. and Tamura, G. (1968). Surfactin, a crystalline peptidolipid surfactant produced by Bacillus subtilis: isolation, characterization and its inhibition of fibrin clot formation. Biochem. Res. Commun., 31, 488-494.
Banat, I.M., Makkar, R.S. and Cameotra, S.S. (2000). Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol., 53, 495-508.
Dearfield, K.L., Cimino, M.C., McCarron, N.E., Mauer, I. and Valcovic, L.R. (2002). Genotoxicity risk assessment: a proposed classification strategy. Mutat. Res., 521, 121-135.
Desai, J.D. and Banat, I.M. (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev., 61, 47-64.
Galloway, S., Aardema, M., Ishidate, M., Ivett, J., Kirkland D., Morita, T., Mosesso, P. and Sofuni, T. (1994). Report from the working group on in vitro tests for chromosomal aberrations. Mutat. Res., 312, 241-262.
Georgiou, G., Liu, S.C. and Sharma, M.M. (1990). Surface active compounds from microorganisms. Biotechnol., 10, 60-65.
Hwang, Y.H., Park, B.K., Lim, J.H., Kim, M.S., Song, I.B., Park, S.C. and Yun, H.I. (2008). Evaluation of genetic and developmental toxicity of surfactin C from Bacillus subtilis BC1212. J. Health Sci., 54, 101-106.
Kanamoto, S., Nagai, S., Ohki, K. and Yasuda, Y. (1995). Study on surfactin, a cyclic depsipeptide. I. Isolation and structure of eight surfactin analogs produced by Bacillus natto KMD 2311. Yakugaku. Zasshi., 115, 756-764.
Kikuchi, T. and Hasumi, K. (2002). Enhancement of plasmidogen activation surfactin C: augmentation of fibrinolysis in vitro and in vivo. Biochem. Biophys. Acta., 1596, 234-245.
Lim, J.H., Park, B.K., Kim, M.S., Hwang, M.H., Rhee, M.H., Park, S.C. and Yun, H.I. (2005). The anti-thrombotic activity of surfactins. J. Vet. Sci., 6, 353-355.
Mulligan, C.N. (2005). Environmental applications for biosurfactants. Environ Pollut., 133, 183-198.
O’Brien, P.J., Hales, B.F., Josephy, P.D., Castonguay, A., Yamazoe, Y. and Guengerich, F.P. (1996). Chemical carcinogenesis, mutagenesis and teratogenesis. Can. J. Physiol. Pharmacol., 74, 565-571.
Organization for Economic Cooperation and Development (OECD, 1997). Test Guideline 473, In vitro Mammalian chromosome aberration test. In: OECD Guidelines for the Testing of Chemicals. OECD Paris.
Park, B.K., Lim, J.H., Hwang, Y.H., Kim, M.S., Song, I.B., Lee, H.G., Han, S.J., Hwang, M.H., Kim, J.W., Park, S.C., Rhee, M.H. and Yun, H.I. (2006). Acute oral toxicity of surfactin C in mice. J. Toxicol. Pub. Health, 22, 453-458.
Rodrigues, L., Banat, I.M., Teixeira, J. and Oliveira, R. (2006). Biosurfactants: potential applications in medicine. J. Antimicrob. Chemother., 57, 609-618.
Singh, P. and Cameotra, S.S. (2004). Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol., 22, 142-146.
Scott, D., Danford, N., Dean, B., Kirkland, D. and Richardson C. (1983). In vitro chromosome aberration assays, Report of the UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, Part 1, Cambridge University Press, Cambridge, UK, pp. 41-64.
Takahashi, T., Ohno, O., Ikeda, Y., Sawa, R., Homma, Y., IGaraishi, M. and Umezawa, K. (2006). Inhibition of lipopolysaccharide activity by a bacterial cyclic lipopeptide surfactin. J. Antibiot (Tokyo), 59, 35-43.