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NOTE

Effect of cobalt and vitamin B_{12} on the production of salinosporamides by *Salinispora tropica*

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NPI-0052 (Figure 1) is a novel, potent proteasome inhibitor[1,2] isolated from a marine actinomycete *Salinispora tropica*.[3] It possesses a broad spectrum of activities against various tumors in animal models,[1,2,4,5] and is currently being evaluated in clinical trials for the treatment of patients with hematologic and solid tumor malignancies.[2] A saline fermentation process for the large scale production of NPI-0052 has been developed to supply NPI-0052 for clinical study. Owing to the structure similarity of NPI-0052 and cometabolites, NPI-0047 (Figure 1) and NPI-2065 (Figure 1), and their close chromatographic elution profiles, the recovery yield of NPI-0052 is highly dependent on the amount of NPI-0047 and NPI-2065 present in the fermentation.

During the development of the defined salt formulations to replace the nondefined synthetic sea salt formulation in supporting the cGMP production of NPI-0052 by *S. tropica* NPS21184, we observed that the production of NPI-0047 in a medium containing the defined salt formulation I was significantly lower than that in a medium containing the synthetic sea salt formulation.[6] Inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) analysis of seven major ion concentrations in the two salt formulations revealed that the defined salt formulation contained a fivefold higher cobalt concentration than the synthetic sea salt formulation.[6] This observation suggested that cobalt may play a role in the production of NPI-0047. In this study, we provide data on the effect of cobalt on the production of NPI-0047 and the two closely related salinosporamides, NPI-0052 and NPI-2065. As cobalt is an essential component of vitamin B_{12}, a coenzyme involved in methylation and carbon skeletal rearrangement reactions,[7] we also examined the effect of vitamin B_{12} on the production of salinosporamides.

MATERIALS AND METHODS

Microorganism

Three *S. tropica* strains, CNB440,[8] CNB476[8] and NPS21184,[9] were used in this study. The CNB440, CNB476 and NPS21184 were deposited with ATCC (the American type culture collection) and assigned the accession numbers ATCC BAA-916 T, PTA-5275 and PTA-6685, respectively.

Growth media and salt formulations

The composition of seed medium SD2, production medium SHY and defined salt formulation I was described by Tsueng *et al.*[6] Synthetic sea salt formulation, Instant Ocean, from Aquarium Systems (Mentor, OH, USA) was used in this study. Defined salt formulation I and Instant Ocean were supplemented with cobalt chloride hexahydrate (Mallinckrodt, Paris, KY, USA) and vitamin B_{12} (cyanocobalamin; Tokyo Kasei Kogyo, Tokyo, Japan), as described in the Results.

Culturing conditions, extraction and HPLC analysis

The culturing conditions, the preparation of fermentation extracts and the analysis of salinosporamides in the extracts by HPLC were described by Tsueng *et al.*[6] For the butyric acid-feeding study, sodium butyrate (Sigma, St Louis, MO, USA) was added to the production culture at a final concentration of 0.9 μM at 47 h followed by the addition of 2 g of Amberlite XAD-7 resin (Sigma, St Louis, MO, USA) after 1 h.

In the synthetic sea salt formulation medium without cobalt or vitamin B_{12} supplement, the production of NPI-0047 was 15 mg l^{-1}, 6.6% of NPI-0052 production (control, Table 1). When the synthetic sea salt formulation was supplemented with cobalt chloride as low as 0.055 μM, the production of NPI-0047 was reduced by 41% to 8.9 mg l^{-1} (Table 1). Maximal inhibition of NPI-0047 production (65%, 5.3 mg l^{-1}) was observed at an added cobalt concentration of 0.22 μM (Table 1). The amount of NPI-0047 produced was 2% of the titer of NPI-0052. Increasing the cobalt concentration to 2.2 μM in the synthetic sea salt formulation did not exert an additional inhibitory effect on the production of NPI-0047 (5.4 mg l^{-1}, Table 1). The addition of cobalt to the synthetic sea salt formulation also decreased the production of NPI-2065 by 30%, and increased the production of NPI-0052 by 16% (Table 1).

The effect of supplementing vitamin B_{12} to the synthetic sea salt formulation on the production of salinosporamides was also

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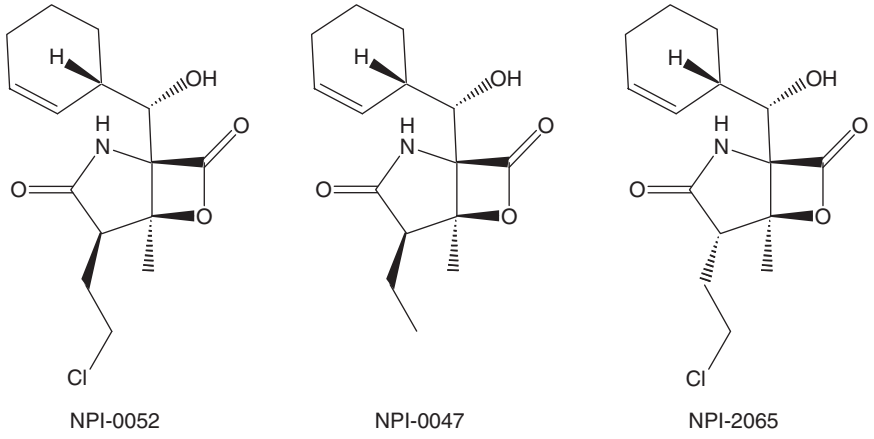

Figure 1 Structure of NPI-0052, NPI-0047 and NPI-2065.

Table 1 The effect of cobalt and vitamin B_{12} on the production of salinosporamides by *Salinispora tropica* NPS21184 in media containing synthetic sea salt or defined salt formulations

Salt formulation	*Additive*	*NPI-0047 (mg l^{-1})*	*NPI-0052 (mg l^{-1})*	*NPI-2065 (mg l^{-1})*
Synthetic sea salt formulation	Control, no addition	15 ± 0.8	228 ± 8	7.8 ± 0.5
	0.055 μM cobalt chloride	8.9 ± 0.9	261 ± 10	5.8 ± 0.2
	0.22 μM cobalt chloride	5.3 ± 0.4	265 ± 8	5.5 ± 0.1
	0.88 μM cobalt chloride	5.4 ± 0.4	274 ± 11	5.3 ± 0.1
	2.2 μM cobalt chloride	5.8 ± 0.3	271 ± 7	5.5 ± 0.2
	0.055 μM vitamin B12	3.7 ± 0.4	269 ± 9	5.6 ± 0.3
	0.22 μM vitamin B_{12}	2.8 ± 0.2	266 ± 9	4.9 ± 0.2
	0.88 μM vitamin B_{12}	2.8 ± 0.3	266 ± 11	5.4 ± 0.2
	2.2 μM vitamin B_{12}	2.5 ± 0.2	247 ± 10	4.9 ± 0.2
Defined salt formulation	Control, no addition	11 ± 0.8	248 ± 6	7.8 ± 0.3
	0.22 μM cobalt chloride	1.2 ± 0.1	292 ± 9	5.4 ± 0.3
	0.88 μM cobalt chloride	1.4 ± 0.1	293 ± 10	6.5 ± 0.3
	0.22 μM vitamin B_{12}	0.96 ± 0.05	275 ± 12	5.8 ± 0.2
	0.88 μM vitamin B_{12}	0.90 ± 0.08	270 ± 9	5.9 ± 0.3

examined and the results are shown in Table 1. Although the effect of vitamin B_{12} on the production of salinosporamides was similar to that of cobalt, vitamin B_{12} exerted a stronger inhibitory effect than cobalt on the production of NPI-0047, maximally inhibiting the production of NPI-0047 by 83%. The production of NPI-0047 was 2.8 mg l^{-1}, 1.1% of the production of NPI-0052, in the vitamin B_{12} salt formulation medium.

We examined the effect of cobalt and vitamin B_{12} at two different concentrations (0.22 and 0.88 μM) on the production of salinosporamides by *S. tropica* strain, NPS21184, in medium containing defined salt formulation. The results are summarized in Table 1. In the defined salt formulation medium without cobalt or vitamin B_{12} supplement, the production of NPI-0047 was 11 mg l^{-1}, 4.5% of the NPI-0052 production. Vitamin B_{12} (92%) exerted a stronger inhibitory effect on the production of NPI-0047 than did cobalt (88%) in the medium containing defined salt formulation. The production of NPI-0047 was 0.9 mg l^{-1}, 0.3% of the production of NPI-0052, in the vitamin B_{12} salt formulation medium. In the cobalt salt formulation medium, the production of NPI-0047 was 1.4 mg l^{-1}, 0.5% of the production of NPI-0052. The production of NPI-0047 in the defined salt formulation medium was lower than that in the synthetic sea salt formulation medium. Cobalt and vitamin B_{12} inhibited the production of NPI-2065 by 17 and 26%, respectively, and increased the production of NPI-0052 by 18 and 11%, respectively.

The effect of cobalt on the production of salinosporamides in the medium containing the defined salt formulation by three *S. tropica* strains, CNB440, CNB476 and NPS21184, was compared, and the results are summarized in Table 2. Although cobalt exerted an inhibitory effect on the production of NPI-0047 in all three *S. tropica* strains, the extent of inhibition was different in each strain. Cobalt inhibited the production of NPI-0047 in CNB440, CNB476 and NPS21184 by 48 (6.8 mg l^{-1}), 52 (8.1 mg l^{-1}) and 89% (1.2 mg l^{-1}), respectively. Cobalt increased the production of NPI-0052 in NPS21184 by 18%, but had no effect on the production of NPI-0052 in CNB440 and CNB476. In CNB476 and NPS21184, cobalt inhibited the production of NPI-2065 by 31% each, but had no effect on the production of NPI-2065 in CNB440. The maximal production of NPI-0052 by NPS21184 in a defined salt formulation medium supplemented with cobalt was 292 mg l^{-1}, approximately threefold higher than the production of NPI-0052 by CNB440 (84 mg l^{-1}) and CNB476 (111 mg l^{-1}).

In our earlier biosynthetic study, we showed that sodium butyrate incorporated into NPI-0047, but not into NPI-0052.[9] Feeding sodium butyrate to cultures of NPS21184 (grown in synthetic sea salt

Table 2 The effect of cobalt and vitamin B_{12} on the production of salinosporamides in defined salt formulation medium by *Salinispora tropica* strains CNB440, CNB476 and NPS21184

	NPI-0047 (mg l^{-1})	*NPI-0052 (mg l^{-1})*	*NPI-2065 (mg l^{-1})*
CNB440, control	13 ± 0.9	100 ± 3	0.8 ± 0.06
CNB440, 0.22 μM cobalt chloride	6.8 ± 0.5	84 ± 2	0.7 ± 0.07
CNB476, control	17 ± 0.7	111 ± 3	1.3 ± 0.10
CNB476, 0.22 μM cobalt chloride	8.1 ± 0.4	111 ± 2	0.9 ± 0.04
NPS21184, control	11 ± 0.8	248 ± 6	7.8 ± 0.30
NPS21184, 0.22 μM cobalt chloride	1.2 ± 0.1	292 ± 9	5.4 ± 0.30

Table 3 Inhibition of butyric acid incorporation into NPI-0047 by cobalt and vitamin B_{12} in *Salinispora tropica* NPS21184

	NPI-0047 (mg l^{-1})	*NPI-0052 (mg l^{-1})*	*NPI-2065 (mg l^{-1})*
Control, no addition	11 ± 0.7	252 ± 9	6.3 ± 0.4
Control+9 μM sodium butyrate	34 ± 3	200 ± 6	4.6 ± 0.2
Control+9 μM sodium butyrate+0.22 μM cobalt chloride	15 ± 0.8	181 ± 5	4.0 ± 0.5
Control+9 μM sodium butyrate+0.22 μM vitamin B_{12}	5.6 ± 0.3	159 ± 6	3.2 ± 0.3

formulation medium) enhanced the production of NPI-0047 by as much as 319% while inhibiting the production of NPI-0052 by 26%.[9] The effect of cobalt and vitamin B_{12} on the incorporation of sodium butyrate into NPI-0047 by NPS21184 grown in a medium containing defined salt formulation was examined. Supplementing the growth medium with sodium butyrate (9 μM) without added cobalt or vitamin B_{12} increased the production of NPI-0047 from 11 mg l^{-1} (control, no sodium butyrate) to 34 mg l^{-1}, an increase of 209% (Table 3). Adding 0.22 μM cobalt and vitamin B_{12} to the butyrate-supplemented cultures decreased the production of NPI-0047 to 15 and 5.6 mg l^{-1}, respectively (Table 3). Vitamin B_{12} exerted a stronger inhibitory effect on the incorporation of sodium butyrate into NPI-0047 than cobalt.

In this study, we show that cobalt and vitamin B_{12} inhibit the production of NPI-0047 through *S. tropica* by examining their effects on the production of NPI-0047 in media containing two different salt formulations. This is an important finding with practical applications to the manufacturing of NPI-0052. The cometabolites, NPI-0047 and NPI-2065, have elution profiles similar to NPI-0052 in both normal and reverse phase chromatography. The effectiveness in separating NPI-0047 from NPI-0052 in the crude extract by flash column chromatography without lowering the recovery yield of NPI-0052 is dependent on the relative ratio of NPI-0047 to NPI-0052. Inclusion of cobalt and vitamin B_{12} at a concentration of 0.88 μM in the defined salt formulation medium significantly reduced the production of NPI-0047 by 87–92% and slightly increased the production of NPI-0052 by 8.9–18% by NPS21184, thereby lowering the ratio of NPI-0047 to NPI-0052 from 4.4 to 0.3–0.5%. Furthermore, cobalt and vitamin B_{12} also slightly reduced the production of the other interfering analog, NPI-2065, by 17–26% in NPS21184. This might improve the recovery yield of NPI-0052 by removing NPI-2065 from NPI-0052 in the subsequent purification step. A simple addition of a metal ion or a cofactor to the salt formulation has significant effect in improving the metabolite production profile without using laborious genetic engineering techniques.

As vitamin B_{12} exerted a stronger inhibitory effect on the production of NPI-0047 than did cobalt, the inhibitory effect of cobalt may be the result of increased production of vitamin B_{12} in the microorganism. Vitamin B_{12} is a coenzyme for the methyltransferase, mediating the terminal step in the biosynthesis of methionine, a common donor of methyl groups through S-adenosylmethionine.[7] Numerous reports on the increase in production of methylated antibiotics and the decrease in production of demethylated antibiotics by the addition of cobalt or vitamin B_{12} to fermentation have been reported.[10–13] All the above methylated antibiotics require methionine as methyl donor. Using [13C-methyl]-methionine in a feeding study experiment, we found that the methyl group of methionine did not incorporate into NPI-0047 and NPI-0052 (data not shown). Although we have not determined the molecular mechanism in inhibiting the production of NPI-0047 by cobalt and vitamin B_{12}, we showed that cobalt and vitamin B_{12} exerted their effects on the inhibition of incorporation of precursor butyric acid into NPI-0047. The slight increase in the production of NPI-0052 might be because of shunting the precursors from the NPI-0047 pathway toward the NPI-0052 pathway.

In this study, we observed that different strains of *S. tropica* respond differently to the effect of cobalt on the production of salinosporamides. *S. tropica* CNB440 is the type strain isolated from a sediment sample by Jensen *et al.*,[8] and its taxonomical characterization has been described by Maldonado *et al.*[14] *S. tropica* CNB476 was isolated from another sediment sample by Jensen *et al.*[8] *S. tropica* NPS21184 is a single colony isolate derived from CNB476 by Tsueng *et al.*[9] Cobalt exerted a significantly stronger inhibitory effect on the production of NPI-0047 in NPS21184 (89%) than that in CNB440 (48%) and CNB476 (52%). We have recently shown that these three *S. tropica* strains are true marine actinomycetes even though they do not require seawater for growth; they have a certain osmotic pressure growth requirement.[15] However, these three strains have different sensitivities to the medium ionic strength for the maintenance of viability and growth;[15] therefore, it is not surprising to observe different responses of the cobalt effect exhibited by these three strains. As there is very limited knowledge of the physiology of marine actinomycetes, further work is required to identify the mechanisms that lead to the different responses to cobalt and medium ionic strength in these *S. tropica* strains.

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