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作品名稱 Expectations for extension of cell life and next generation anticancer drugs by using secondary metabolites of actinomycetes

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作者照片

Abstract

Inhibitory effects of the secondary metabolite of actinomycete were examined on cell cycle of the yeasts of *S. pombe* and *S. cerevisiae*. The secondary metabolite was obtained from cultivation of the actinomycete isolated from the soil of Owakudani in Hakone, Japan. The fifth fraction of the secondary metabolite by ODS column separation (HK-T5), which was soluble to pure methanol, was used in the present experiments. The HK-T5 brought about the delay of forming colonies of *S. pombe* for about 11 days compared to that cultivated without the HK-T5. The delay of the colony formation was longer for the *S. pombe* cultivated with more amount of the HK-T5. The cultivation with HK-T5 also brought about the extension of the lifespan of the *S. pombe* for more than 10 weeks in a liquidus medium. The cell life recovered the ordinary manner by removal of the HK-T5, meaning that the activities of the HK-T5 is reversible. These facts confirm the suppression of cell cycle, and the delay of cell growth by the HK-T5. These phenomena were similarly observed for *S. cerevisiae*.

Comparison of the action of HK-T5 with hydroxyurea, which is an anticancer drug inhibiting the cell cycle at S phase, clarified that the inhibitory action of HK-T5 worked at the phase earlier than S phase. The combined effects of HK-T5 on the cell cycle were evaluated with triamcinolone acetonide (TA), or aspirin, the former of which is a drug synchronizing cancer cells in S phase, and the latter keeping human cells in G1/G0 phases. The combined use of HK-T5 with TA synchronized the cells at the phase slightly proceeding from G1 to S phase without toxicity. On the other hand, the combined use with aspirin made the inhibitory effect of HK-T5 inactive.

Hence, the HK-T5 is attractive as a drug for the extension of cell lifespan, and anticancer therapy.

1. Introduction

Many of the conventional anticancer drugs affect the S, G2 and M phases in the cell cycle. However, this causes the undesired side-effects to the healthy cells in spite of the effective action to the cancer cells. On the other hand, a cancer cell goes through the cell cycle in the uncontrollable manner without shifting phases from G1 to G0 whereas most of the ordinary human cells stay in G0 phase. Therefore, it is desirable to develop the next-generation anticancer drug that affects the G1/G0 phases[1] (Fig. 1).

In 2017, the authors found that a secondary metabolite of the actinomycete isolated from the soil of Owakudani in Hakone, Japan, temporarily inhibited the growth of fungal without any activity to prokaryotic. In this study, it was aimed to evaluate the inhibitory effect of the secondary metabolite on the growth of yeast so as to consider its applicability to the drug for expansion of cell life and anticancer therapy.

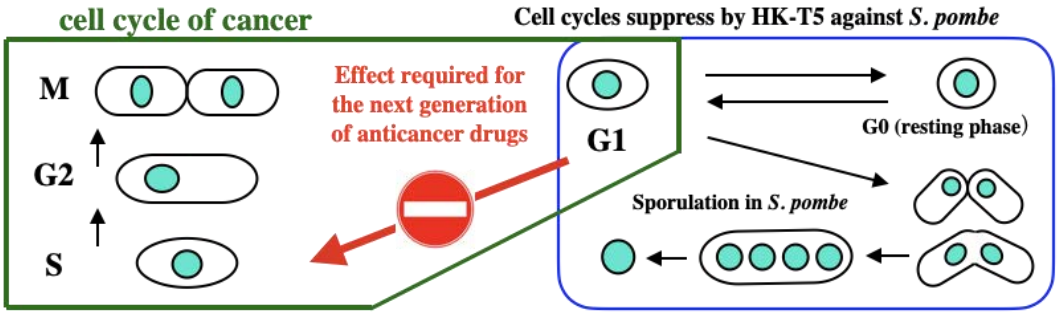

Fig. 1 Cell cycle (human cells, cancer cells and *S.pombe*) and the presumed cell cycle in which HK-T5 acts

2. About *Streptomyces* HK-T and its secondary metabolites HK-T5

The actinomycete isolated from the soil of Owakudani in Hakone was revealed as an unknown species of the genus *Streptomyces* by 16S rRNA gene sequence analysis, and then was referred to as HK-T (Fig. 2). The HK-T was cultured on an agar medium containing soybean flour and glucose as nutrients. The secondary metabolites were then extracted by methanol. After the centrifugal separation and drying treatment, the extract was then separated into the five fractions by ODS column from water-soluble to fat-soluble components. Hereafter, the fifth fraction, which was eluted by methanol, was referred to as HK-T5 (Fig. 2).

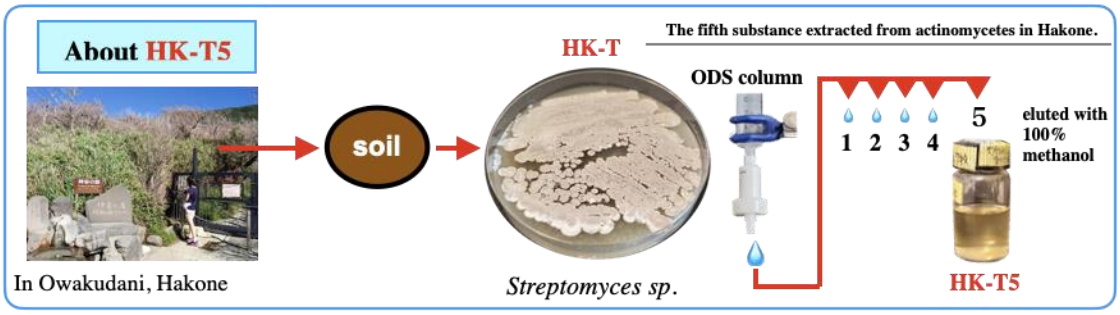

Fig. 2 Isolation and cultivation of HK-T from soil and purification of secondary metabolites by ODS column

Unique characteristics of HK-T5

As preliminary experiments, paper discs were impregnated with HK-T5 and were subjected to the activity tests for *S. pombe*, *S. cerevisiae* and *Aspergillus* molds in YPD (Yeast extract, Peptone, Dextrose) and RG (Raisin extract, Glucose, agar) media. For the candidates, including the HK-T5, of the anticancer drugs we found, an inhibition circle was formed, meaning their bactericidal action to the yeasts and molds. However, for only the HK-T5, the yeasts and molds started to regrow at the inhibition circle, indicating that the inhibitory action of HK-T5 reversibly worked to the cell proliferation (Fig. 3).

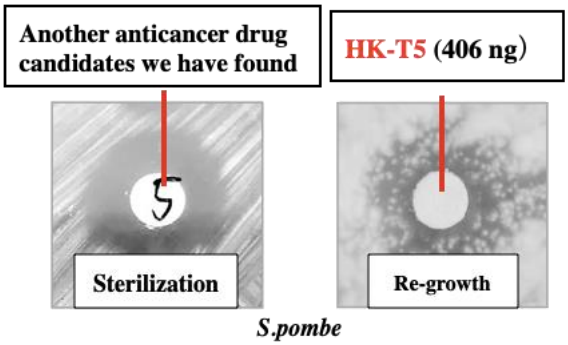

Fig. 3 Comparison of the suppression of cell division of HK-T5 and other candidate compound

3. Experimental and results

3-1 Growth delay of yeast by HK-T5

S. pombe was cultivated at 28 ℃ for 24 h in 200 μL of an RG liquidus medium (A), or in that with addition of 406 ng of HK-T5 (B). After the cultivation, the content of A or B was dropped onto an agar medium, and then the growth of colonies was observed with time. The experimental results showed that the *S. pombe* cultivated without HK-T5 began to form colonies after about 24 h, followed by the deterioration after about 2 weeks via sufficient growth until about 72 h. On the other hand, for the *S. pombe* cultivated with HK-T5, the colony formation initiated after about 72 h, and attained to the sufficient growth until about 2 weeks (Fig. 4). In other words, the HK-T5 brought about the delay of cell growth for about 11 days on the basis of visual observation. The delay of the cell growth was compared among the different amounts of HK-T5 added of 0, 406, and 609 ng. It was clarified that the larger amount of HK-T5 gave rise to the longer delay of the cell growth (Fig. 4). These facts indicate that the HK-T5 has an ability to prolong the lifespan of the yeast. It can also be inferred that the inhibitory activity is reversible since the removal of the HK-T5 restored the cell growth of the yeast.

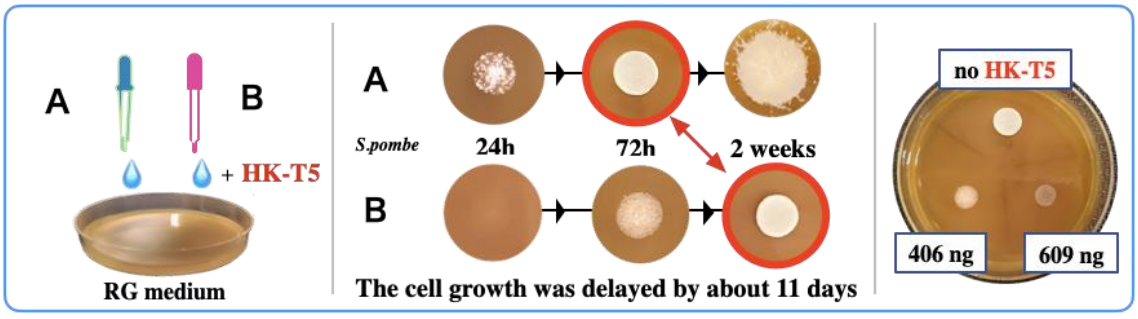

Fig. 4 Temporal mitotic inhibition by HK-T5 addition and difference in inhibition by concentration

3-2 Extension of cell life in yeast by HK-T5

S. pombe cultivated for 3 weeks in the same liquidus media of A and B was also dropped onto an agar medium, and then the growth of colonies was observed with time. The experimental results clearly showed that few colonies formed on the medium for the *S. pombe* cultivated without HK-T5 whereas the obvious colony formation was detected for that cultivated with HK-T5 (Fig. 5). This means that the cultivation with HK-T5 keeps the *S. pombe* living while it was extinguished within 3 weeks by the cultivation without HK-T5.

Additional experiments revealed that 812 ng of HK-T5, which is the twice amount of HK-T5 in B, also maintains the bioactivity of the *S. pombe* even after the cultivation for 13 weeks (Fig. 5). Further experiments confirmed this activity of HK-T5 to *S. cerevisiae*. These results support the hypothesis that the HK-T5 possesses the ability of extension of cell lifespan for the yeast.

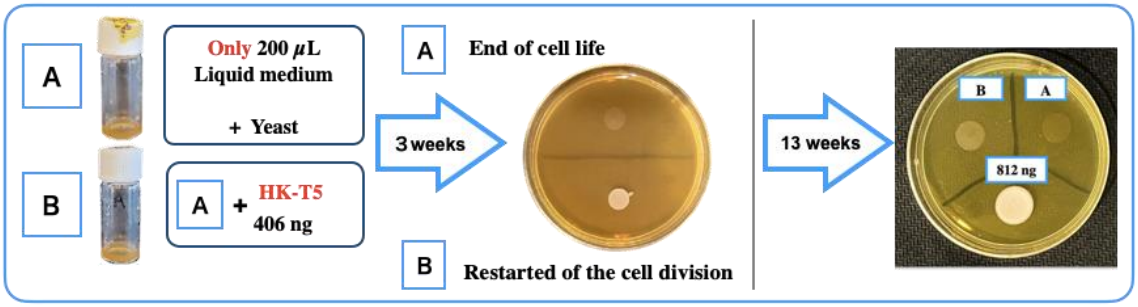

Fig. 5 Extending cell lifespan by adding **HK-T5**

3-3 Microscopic observation

Microscopic observation was periodically conducted for the *S. pombe* cultivated in 200 μL of an RG liquidus medium, and a YPD liquidus medium where HK-T5 was added of 0 ng (1), 406 ng (2), or 609 ng (3) (Figure. 6-1) (Figure. 6-2). For the cultivation without HK-T5, the cell division, and the cell proliferation actively occurred, providing the mixture of the cells in all the phases of G1/G0, S, G2, and M. On the other hand, the cultivation with 406 ng of HK-T5 gave rise to the less of the cell division, and, in particular, the smaller amount of the cells in the M phase. Furthermore, when cultivated with 609 ng of the HK-T5, the *S. pombe* stopped the cell division, and also stopped the cell proliferation, remaining the cells in the G1/G0 phases, or, the sporulation phase. This observation clarified that the larger amount of HK-T5 suppressed more the cell cycle, and delayed longer the cell growth. Hence, it is suggested that the HK-T5 possibly has an ability as a next-generation cancer drug targeting the termination of cell cycle at the G1 phase.

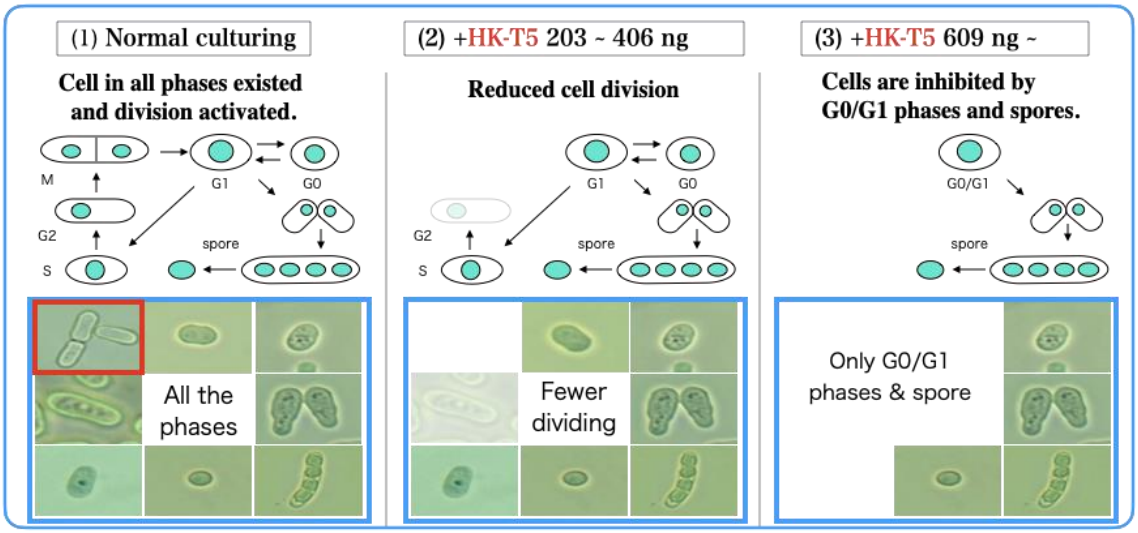

Fig.6-1 Inhibition of cell cycle by HK-T5 concentration

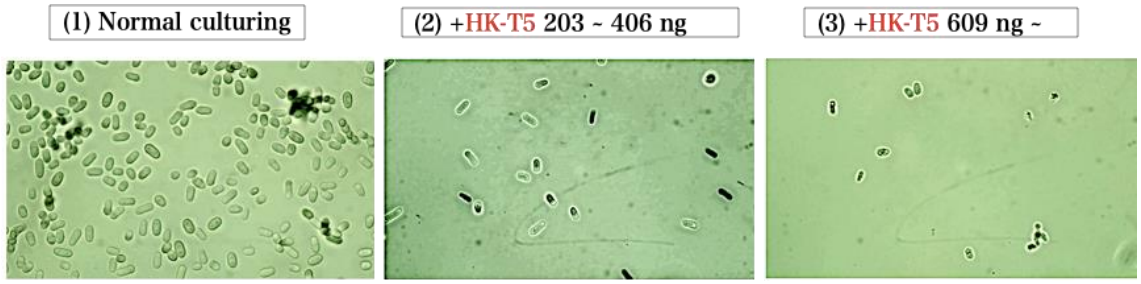

Fig.6-2 Full screen capture

3-4 Comparison of HK-T5 with hydroxyurea, an anticancer drug

Hydroxyurea (HU) is known as an anticancer drug that inhibits the cell cycle in the S phase of *S. pombe*[2]. Thus, the supplemental effect of HK-T5 on the cell cycle was compared with that of HU in order to confirm the activable stage of the HK-T5 in the cell cycle. Three different amounts of HU of 212, 424, and 848 μg were employed to provide the dose-dependent delay of the cell growth (Fig. 7-1). Microscopic observation showed that the cultivation with 424 μg of HU for 96 h decreased the amounts of the cells in M2 phase. Some of the cells became abnormally transparent, and then the cells in M phase were deformed and destroyed. The state of most of the cells looked like the cells further progressing the phases than those derived from the cultivation with 609 ng of the HK-T5 (Fig. 6-1) (Fig. 7-2). However, the cells were never deformed and destroyed by the cultivation with the HK-T5. These results indicate that the HK-T5 suppressed the cell cycle at the earlier stage than S phase. Hence, the HK-T5 is considered to be a candidate of the next-generation anticancer drug.

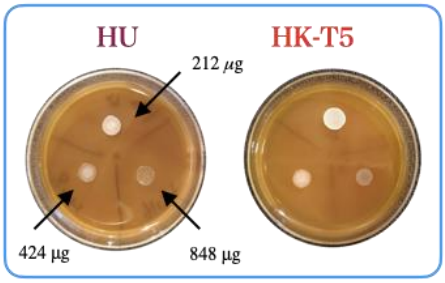

Fig. 7-1 HU & HK-T5
Growth delay by concentration

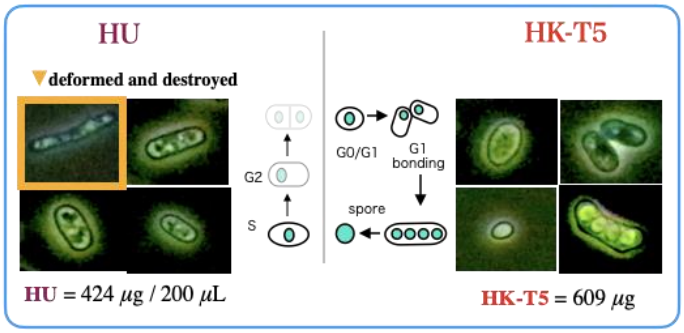

Fig.7-2 Comparison of inhibition cycles by HU and HK-T5

4. Verification of the combination of HK-T5 and other compounds

In general, many of the anticancer drugs are used combinatorially with other drugs. For example, the previous study reported that the Rapamycin, which is an immunosuppressant derived from actinomycetes, works effectively to cancer cells when it is used in combination with the Doxorubicin[3]. Hence, in this study, the effects of the combined use of HK-T5 with triamcinolone acetonide (TA), and that with aspirin were examined on the cell cycle of yeast.

4-1 The combination effect of HK-T5 and triamcinolone acetonide

Since it is known that TA synchronizes the cancer cells in the S phase[4], the combined use of HK-T5 with TA is expected to show a synergistic effect on the cell cycle. Therefore, some experimental runs were conducted of cultivation of *S. pombe* at 28 ℃ in 200 μL of RG medium containing 202 μg of TA and 0, 203, and 406 ng of HK-T5. The experimental results clarified that the combined use of TA with HK-T5 brought about the suppression of the cell growth (Fig. 8-1). It was also seen that the larger amount of HK-T5 delayed the cell growth longer. Furthermore, the cells of *S. pombe* cultivated in the medium containing 220 μg of TA and 203 ng of HK-T5 for 96 h were synchronized to each other at the phase slightly proceeding from G1 phase to S phase (Fig. 8-2). This behavior was similar to that for the cells cultivated in the medium only containing 212 μg of HU. On the other hand, while the cultivation with only the HU brought the deformation and/or destruction of the cell shapes due to the toxicity of HU whereas that with the combined drugs of TA and HK-T5 hardly did so. It was also revealed that the effects of the combined drugs on the cell cycle were reversible. These facts mean that TA does not have toxicity to the yeast and can be used as a conditioning drug to shift the affectable phase of HK-T5 from G1/G0 to that closer to S phase.

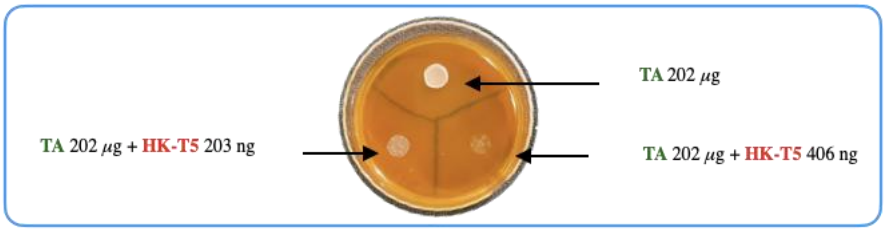

Fig. 8-1 Growth delay by combined use of HK-T5 and TA

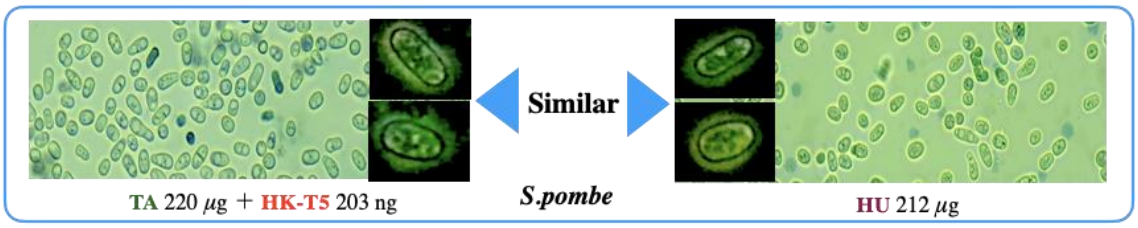

Fig. 8-2 Comparison of cell morphology between HK-T5 and TA-infused yeast and HU-infused yeast

4-2 The combination effect of HK-T5 and aspirin

Since it is known that aspirin keeps human cells in the G0/G1 phases[5], the combined use of HK-T5 with aspirin is also expected to show a synergistic effect on the cell cycle. However, some experiments made clear that the colonies were similarly formed from the yeast cultivated in the media containing HK-T5 with different amounts of aspirin of 45 to 360 μg, meaning that the loss of the effect of the cell-growth-delay by HK-T5 (Fig. 9). This phenomenon was observed for both of *S. pombe* and *S. cerevisiae* in the same manner, giving the difficult determination for suppression of cell cycle by microscopic observation. These facts also provide that the aspirin can work as a conditioning drug for the HK-T5.

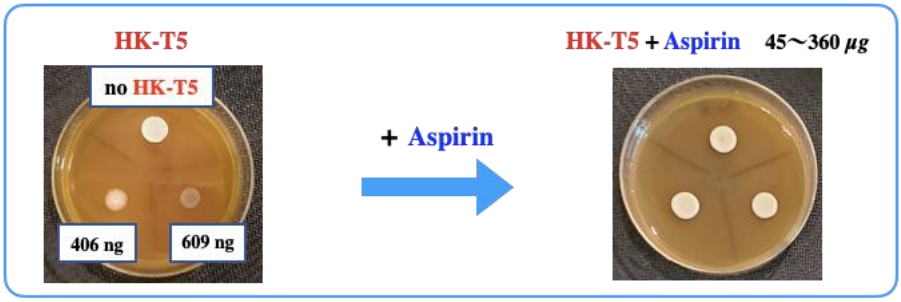

Fig. 9 Loss of the effect of the growth delay of HK-T5 by concomitant use of aspirin

5. Summary and discussions

HK-T5 inhibited the cell division for both the yeasts of *S. pombe* and *S. cerevisiae*, and reversibly suppressed the cell cycle at around the G1/G0 phases. Since the cells suffering the inhibitory cell cycle

sometimes shifted the phases from G1 to sporulation, the HK-T5 is considered to temporarily make the cells close to the state of the lack of nutrient or to inhibit the action of substances such as CDK and so on which are necessary for a cell to shift the phases from G1 to S[(6)]. Triamcinolone acetonide and aspirin are quite attractive for the agents in the determination of the action mechanisms because they showed promise as a modulating drug for the action of the HK-T5 and are also gentle to human body.

6. Future works

The followings are expected to as future works needed.

1. Validation of the activity of HK-T5 of the yeast of laboratory strains
2. Determination of the molecular structure of HK-T5
3. Comparison of the activities of HK-T5 with other known drugs
4. Validation of the activities of HK-T5 in mammalian and human cells together with the several kinds of cancer cells
5. Determination of the detailed mechanisms of action of HK-T5 to cells

7. Acknowledgment

We are grateful to professor Taro Nakamura of Osaka City University for his generous comments and suggestions.

8. Reference

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【評語】070007

In this study titled " Expectations for extension of cell life and next generation anti-cancer drugs by using secondary metabolites of actinomycetes", the two students identified potential secondary metabolites from an unknown actinomycete and demonstrated that the potential metabolite HK-T5 can inhibit cell growth likely through interfering with the cell cycle at the S phase. Overall, we think that the students have conducted a huge amount of work and have devoted a long period of time (almost 5 years) to this project, showing that they are very passionate about their research. Although the communication between the students and us was not perfect due to students' English proficiency, we could understand most of their presentations well. In summary, we would like to encourage these two students to follow their passion and continue their reach to identify the structure of this interesting compound in the future.