Effects of BMI-1026, A Potent CDK Inhibitor, on Murine Oocyte Maturation and Metaphase II Arrest

Tae-Saeng Choi†

Department of Microbiology, College of Medicine, Dankook University, Cheonan 330-714, Korea

ABSTRACT

Previous studies have shown that BMI-1026 is a potent inhibitor of the cyclin-dependent kinases (cdk). In cell culture, the compound also arrests G2/M strongly and G1/S and S weakly. Two key kinases, cdk1 (p34cdc2 kinase) and mitogen-activated protein (MAP) kinase (erk1 and 2), perform crucial roles during oocyte maturation and, later, metaphase II (MII) arrest. In mammalian oocytes, both kinases are activated gradually around the time of germinal vesicle breakdown (GVBD) and maintain high activity in eggs arrested at metaphase II. In this study, we examined the effects of BMI-1026 on GVBD and MII arrest in mouse oocytes. BMI-1026 inhibited GVBD of immature oocytes and activated MII-arrested oocytes in a concentration-dependent manner, with more than 90% of oocytes exhibiting GVBD inhibition and MII activation at 100 nM. This is approximately 500~1,000 times more potent than the activity reported for the cdk inhibitors roscovitine (~50 μM) and butyrolactone (~100 μM). Based on the results of previous *in vitro* kinase assays, we expected BMI-1026 to inhibit only cdk1 activation in oocytes and eggs, not MAP kinase. However, in our cell-based system, it inhibited the activity of both kinases. We also found that the effect of BMI-1026 is reversible. Our results suggest that BMI-1026 inhibits GVBD and activates MII-arrested oocytes efficiently and reversibly and that it also inhibits both cdk1/histone H1 kinase and MAP kinase in mouse oocytes.

(Key words : CDK-1, GVBD, Oocyte activation, MAP-kinase, BMI-1026)

INTRODUCTION

In most vertebrates, two key kinases, cdk1 and mitogen-activated protein (MAP) kinase (Erk 1 and 2), perform crucial roles during oocyte maturation and, later, the metaphase II (MII) arrest of ovulated eggs. Both kinases are activated gradually around the time of germinal vesicle breakdown (GVBD) during oocyte maturation and maintain high activity at the arrest of eggs in metaphase II. Many studies have attempted to determine their distinct biological functions, especially as highly sustained MAP kinase activity is specific to oocytes in meiosis (Choi *et al.*, 1991; Sobajima *et al.*, 1993; Verlhac *et al.*, 1994; Liu *et al.*, 1998; Motlik *et al.*, 1998; Kubelka *et al.*, 2002a).

The *mos* protooncogene encodes a serine/threonine kinase, which is meiosis specific and an upstream regulator of MAP kinase. The activity of cytostatic factor (CSF), which maintains MII arrest and is composed of Mos/MAP kinase, may be responsible for cyclin B stabilization (Maller *et al.*, 2002; Huo *et al.*, 2004). Oocytes of *mos*-deficient mice do not exhibit MAP kinase activity, but do activate cdk1 during oocyte maturation. These oocytes show abnormal spindle formation, asymmetric division in meiosis I, and parthenogenetic activation resulting from the failure to sustain cdk1 activity (Hashimoto *et al.*, 1994; Choi *et al.*, 1996a,b; Verlhac *et al.*, 1996).

BMI-1026 is a new class of Cdk inhibitors, which is synthesized aryl aminopyrimidines substituted with additional aromatic heterocycles (Seong *et al.*, 2003). Previous *in vitro* kinase assays have shown that BMI-1026 is a potent inhibitor of cdk1 (1.2 nM), cdk2 (4.3 nM), cdk5 (6.9 nM), but does not significantly inhibit PKA, PKC-delta, or Erk1. In cultured cell lines, the compound also arrested G2/M strongly and G1/S and S weakly (Seong *et al.*, 2003).

In this study, we investigated the effects of BMI-1026 on oocyte maturation and egg activation.

MATERIALS AND METHODS

Collection of Oocytes and Eggs

The mice used in this study were B6C3F1 females, 3~4 weeks of age. To obtain immature oocytes, female mice were intraperitoneally injected with 5 IU pregnant mare serum gonadotropin (PMSG). After 48 hr, we ob-

* This study was conducted by the research fund of Dankook University in 2005.

† Corresponding author : Phone: +82-41-550-3872, E-mail: tschoi@dankook.ac.kr

tained cumulus-enclosed oocytes from large follicles in their ovaries using sharp needle punctures under a dissecting microscope. The immature oocyte collection media was supplemented with 0.1 mM 3-isobutyl-1-methyl-xanthin (IBMX) to inhibit the resumption of meiotic maturation (Choi *et al.*, 1991). To obtain ovulated eggs, female mice were injected with 5 IU PMSG and then, 45~48 hr later, with 5 IU of human chorionic gonadotrophin (hCG). Ovulated oocytes were collected from the ampulla of oviducts 14~16 hr after hCG injection.

Culture of Oocytes and Eggs

The immature oocytes were washed in IBMX-free medium and then cultured. Only oocytes enclosed within complete cumulus cells were collected, pooled in 0.1 mM isobutylmethylxanthine (IBMX)-supplemented control medium, and subsequently cultured in control medium with or without BMI-1026 at the indicated concentration for 5 hr. BMI-1026 was prepared as 2 mM stock solution in dimethylsulfoxide (DMSO) and keep at −20℃ until use. Oocytes were fixed and stained with 4'.6-diamidino-phenylindol (DAPI), and then the occurrence of GVBD was evaluated under the fluorescence microscope.

To determine whether the BMI-1026 reversibly inhibit GVBD in immature oocytes, the GV-oocytes were cultured in 200 nM BMI-1026 for 5 hr. Then, a half of the oocytes was washed and transferred to control medium, while the other half was continuously cultured with BMI-1026. The occurrence of GVBD was assessed for 2 hr at 30-min intervals.

For the ovulated oocytes, cumulus cells surrounding the eggs were freed by incubation for 2 to 3 min in 0.1% hyaluronidase (type I-S; Sigma, St. Louis, MO, USA) in culture medium at 38.5℃ in a humidified atmosphere of 5% CO_2 and 95% air. The cumulus-free oocytes were rinsed three times in culture medium and cultured with 25, 50, 100, or 200 nM of BMI-1026 in a plastic culture dish (35 × 10 mm; NUNC, Rochester, NY, USA) for 5 hr. We also tested the effects of ethanol activation, which is generally used for egg activation, to compare with BMI-1026. Ovulated eggs treated with 7% ethanol (E-OH) in culture for 7 min at room temperature. The activated eggs were then rinsed three times in culture medium before use.

Histone H1 and MBP Double-Kinase Assay

Histone H1 kinase and Myelin basic protein (MBP) kinase activities were represented by Maturation promoting factor (MPF) and MAP kinase activities, respectively. Fifteen denuded oocytes were lysed in 3 μl kinase assay buffer (50 mM Tris-HCl [pH 7.5], 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 80 mM beta-glycerolphosphate, 0.2 mM sodium orthovanadate, 1 mM PMSF, 10 mM leupeptin, 1 mM pepstatin, 10 mM aprotinin) and frozen at −70℃ until use. After twice freezing and thawing, the lysates were added to 3 μl of 2 μM cAMP-dependent protein kinase inhibitor peptide (Sigma, catalog no. p3294), 3 μl of 3 mg/ml histone H1 (Sigma), 3 μl of 5 mg/ml MBP (Sigma), and 3 μl of 5 mM cold ATP (Sigma). Finally, the kinase reaction was carried out in ~20 μl of kinase assay buffer, including 15 oocytes containing 555 kBq of [gamma-32P] ATP (NEN, Wellesley, MA, USA), for 30 min at 37℃. The kinase reactions were terminated by adding 5 μl of 5X Lammli sample buffer to each lysate, which was then denatured by boiling for 5 min. The samples were analyzed by electrophoresis in 15% SDS-PAGE followed autoradiography (Knockaert *et al.*, 2002).

Evaluation of GVBD, Egg Activation and 2nd Polar Body Extrusion

At the end of the culture, oocytes freed cumulus cells with hyaluronidase were fixed with 1.8% paraformaldehyde in PBS for 40 min at room temperature (RT) and stained with DAPI at RT to visualize the DNA (Choi *et al.*, 1996a). The stained oocytes were observed under a Zeiss fluorescence microscope (Carl Zeiss, Jena, Germany) to evaluate GVBD or pronuclei. Eggs with one or two pronuclei were evaluated as parthenogenetic activated eggs. Twenty oocytes were tested at each time point, and the experiment was repeated three times. To evaluate second polar body extrusion, we removed the first polar body of ovulated oocytes mechanically by pipetting after removing the zona pellucida using acidic Tyrode's solution (Sigma, pH 2.5). The oocytes were activated by E-OH or cultured with BMI-1026. The number of eggs with a second polar body was counted at the indicated times under the microscope.

Statistical Analysis

Each experiment was replicated at least 3 times. Statistical differences between groups were analyzed using one way ANOVA. Data shown as mean ± SEM were calculated.

RESULTS

Effect of BMI-1026 on GVBD of Immature Oocytes

At a concentration of 200 nM, BMI-1026 completely inhibited GVBD. At concentrations of 100, 50, and 25 nM, GVBD was inhibited 95, 31, and 12%, respectively (Fig. 1). Although our oocytes were fixed after 5 hr of culture, the inhibitory effects of BMI-1026 persisted for 24 hr (data not shown).

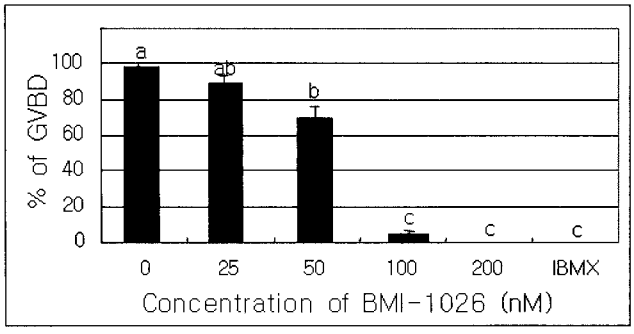

Fig. 1. Effect of BMI-1026 on immature mouse oocyte GVBD. The data show the means ± SEM (n = 4). Different letters indicate significant differences (p<0.05).

Effect of BMI-1026 on Activation of Ovulated Eggs

At a concentration of 200 nM, all of the tested oocytes were activated. At concentrations of 25, 50, and 100 nM, oocyte activation was 57, 64, and 91%, respectively (Fig. 2). The number of oocytes extruding the second polar body was counted at 30 min, 1 hr, 1.5 hr, and 2 hr. As shown in Fig. 3, 52% of oocytes treated with BMI-1026 extruded the second polar body within 30 min, but ethanol treatment activated only 12% of the oocytes.

After demonstrating that BMI-1026 very efficiently inhibited immature oocyte GVBD and induced egg activation (Fig. 1 and 2), we investigated histone H1 kinase and MAP kinase activities using a double-kinase assay method.

Effect of BMI-1026 on Histone H1 and MAP Kinases Activation

During normal oocyte maturation, histone H1 kinase and MAP kinase activity gradually increased for ~7 hr, but BMI-1026 inhibited MAP kinase activation as well as histone H1 kinase, like the IBMX-supplemented control treatment (Fig. 4a, b).

During egg activation induced by either BMI-1026 or ethanol treatment, the activity of both kinases decreased over 7 hr (Fig. 4c, d). The time frame for the inactivation of MAP kinase was very similar for both BMI-1026 and ethanol treatments, but histone H1 kinase was inactivated more rapidly in BMI-1026-treated eggs than in ethanol-treated eggs. The rapid extrusion of the second polar body in eggs treated with BMI- 1026 (Fig. 3) may reflect this immediate inactivation of histone H1 kinase activity.

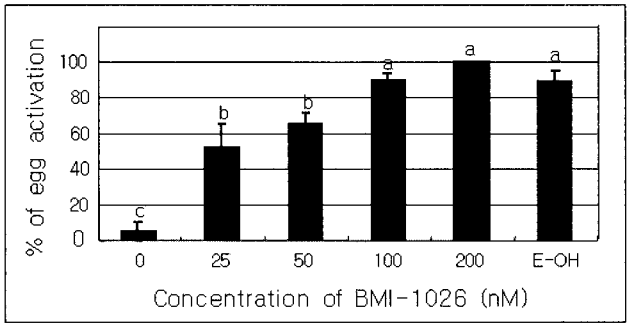

Fig. 2. Effect of BMI-1026 on the activation of ovulated eggs. The data show the means ± SEM (n = 4). Different letters indicate significant differences (p<0.05).

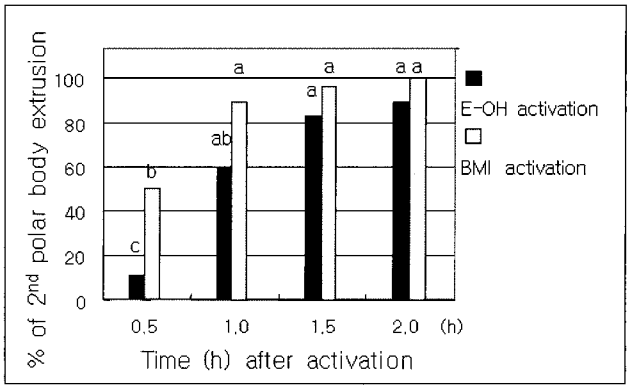

Fig. 3. Effect of BMI-1026 on second polar body extrusion. The data show the means from four replicates, using a total of 80 eggs. The data show the means ± SEM (n = 4) and different superscript within a column were significantly different (p<0.05).

Reversibility of BMI-1026 on GVBD Inhibition

During the culture with BMI-1026, GVBD of the immature oocytes was more or less completely inhibited. One hour after releasing from the culture with BMI-1626 (6 hr of initial culture), however, 83% of the oocytes underwent GVBD (Fig. 5).

DISCUSSION

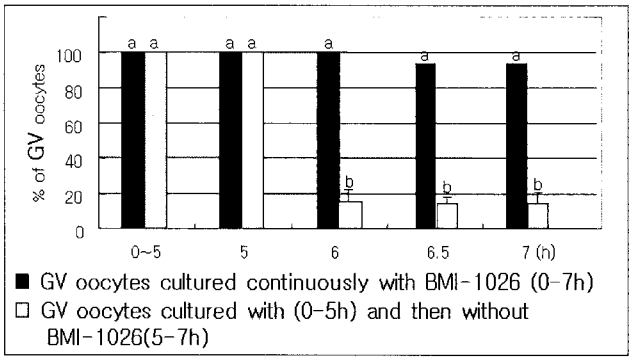

Fig. 5. BMI-1026 reversibly inhibits GVBD of immature mouse oocytes. GV-oocytes were cultured with 200 nM BMI-1026 for 5 hr, then, half of the oocytes (▨) were washed and transferred to control medium, while the other half (■) were continuously cultured with BMI-1026. The data show the means from three replicates, using a total of 80 eggs and the column with different superscripts were significantly different (p<0.05).

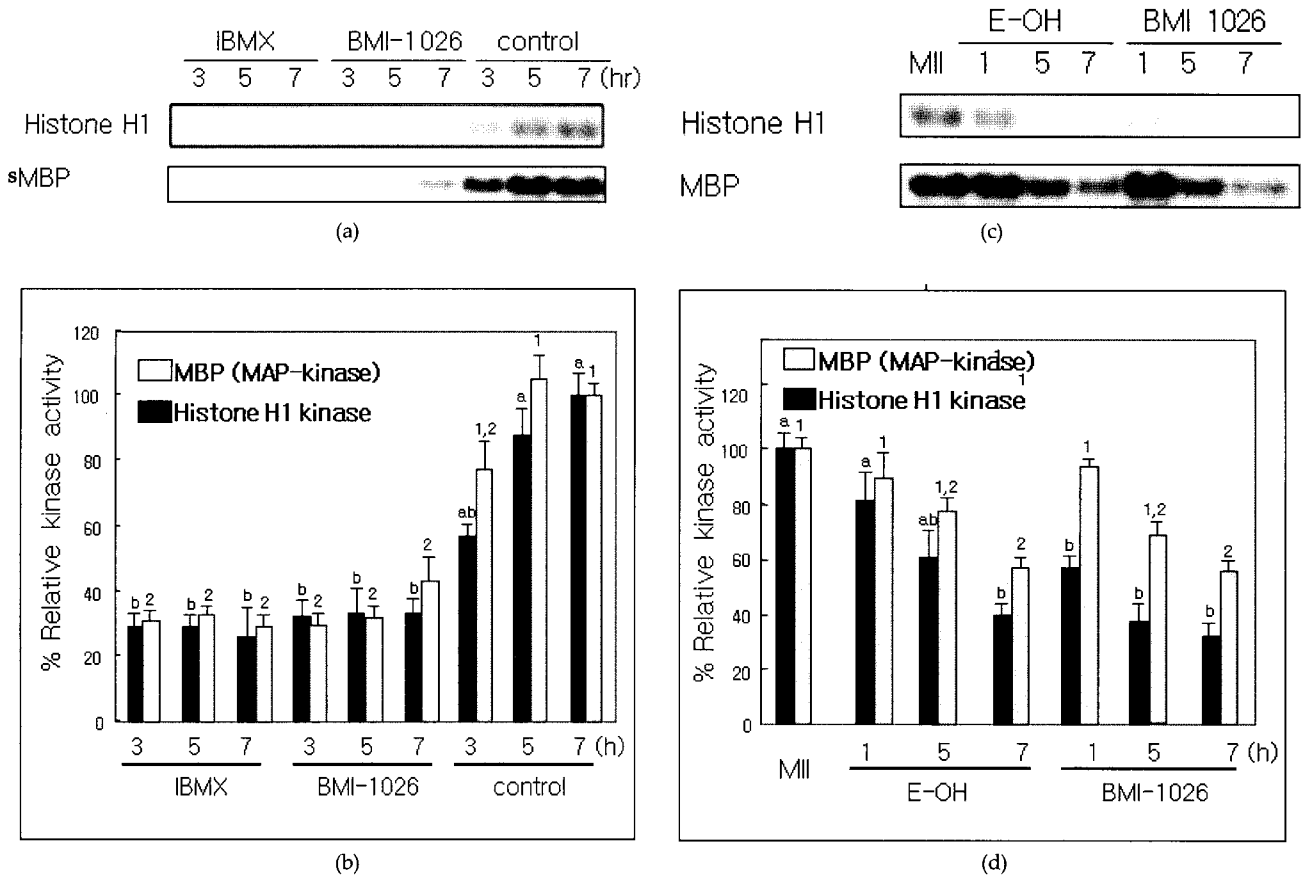

Fig. 4. Effect of BMI-1026 on histone H1 and MAP kinase activity. Histone H1 kinase and MAP kinase activity in maturing mouse oocytes (a). Relative kinase activity (b). In maturing oocytes (MI), the activity of both kinases at 7 hr was designated 100%. Histone H1 kinase and MAP kinase activities in ovulated eggs (c). Relative kinase activity (d). The activity of both kinases in MII (ovulated eggs) was designated 100%. Each histone H1 kinase and MAP- kinase activity was analyzed respectively and represent with different superscripts (alphabet or number) ($p<0.05$).

Here, this study shows that BMI-1026 can arrest mouse oocyte GVBD and effectively activate ovulated eggs. These effects are correlated with the inhibition of MPF/histone H1 kinase.

Numerous studies have shown that cdk1 inhibitors block GVBD and induce the parthenogenetic activation of ovulated eggs. The effective dose of roscovitine or olomoucine to inhibit immature oocyte GVBD and activate ovulated eggs has been reported as 40~200 μM (Meijer *et al.*, 1997; Kubelka *et al.*, 2000; Krischek and Meinecke, 2001; Kubelka *et al.*, 2002b; Ju *et al.*, 2003; Vigneron *et al.*, 2004). This study showed that BMI-1026, with an effective dose of about 50 nM, is a very potent inhibitor of cdk1, compared to roscovitine or olomoucine.

Previous *in vitro* kinase assays have shown that BMI-1026 is a potent inhibitor of cdk1, cdk2, and cdk5, but not of other mitotic kinases, such as erk1, aurora A, and Plk1 (Seong *et al.*, 2003). However, the results of cultured cell studies have shown that BMI-1026 induces G2/M arrest and is a more potent inhibitor of cdk1 than of cdk2, which is required for the cell cycle G1/S transition (Seong *et al.*, 2003). The *in vitro* kinase assay by Seong *et al.* (2003) showed that the selectivity of BMI-1026 against cdk1 and MAP kinase was more than 6,000 times (IC50:cdk1, 8.2 nM; MAP kinase 50 μM). From this previous study, we expected BMI-1026 did not inhibit MAP kinase but cdk1. However, in oocyte system it inhibited both cdk1 and MAP kinase. One possibility is that BMI-1026 can inhibit not only cdk1 but also MAP-kinase *in vivo*. Actually, purvalanol which is selective cdk inhibitor could inhibit cdks and MAP-kinase in intracellular system (Knockaert *et al.*, 2002).

BMI-1026 induced egg parthenogenetic activation efficiently and quickly, compared to ethanol. More than 50% of the eggs treated with BMI-1026 extruded the second polar body within 30 min (see Fig. 3). This speedy progress correlated with histone H1 kinase activity and may depend on the direct inhibition of cdk1,

not cyclin B degradation. In fact, the release of BMI-1026 at an early stage of the activation process (probably before cyclin B degradation) induced the recondensation of chromosomes and formation of the metaphase II spindle (data not shown).

This study also found that the effect of BMI-1026 is reversible, in that GVBD was induced in oocytes cultured in medium free of BMI-1026 after having had GVBD arrested by culture in BMI-1026 (see Fig. 5). This reversibility of BMI-1026 was also confirmed by the experiments of making haploid or diploid embryos from parthenogenetic activation using BMI-1026 of ovulated eggs (data not shown).

In conclusion, BMI-1026 inhibits GVBD of immature mouse oocytes and activated ovulated eggs very efficiently due to cdk1/Histone H1 kinase inhibition. Also, BMI-1026 inhibits MAP kinase activation during oocyte maturation or egg activation. Additionally, this compound is reversible. Taken together, BMI-1026 is a very useful material for study of oocyte maturation and egg activation comparable to previous cdk1 inhibitors.

ACKNOWLEDGEMENT

Author thanks Dr. H. Chung for providing BMI-1026.

REFERENCES

1. Choi T, Aoki F, Mori M, Yamashita M, Nagahama Y, Kohmoto K (1991): Activation of $p34^{cdc2}$ protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. Development 113:789-795.
2. Choi T, Fukasawa K, Zhou R, Tessarollo L, Borror K, Resau J, Vande Woude GF (1996a): The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. Proc Natl Acad Sci USA 93:7032-7035.
3. Choi T, Rulong S, Resau J, Fukasawa K, Matten W, Kuriyama R, Mansour S, Ahn N, Vande Woude GF (1996b): Mos/mitogen-activated protein kinase can induce early meiotic phenotypes in the absence of maturation-promoting factor: a novel system for analyzing spindle formation during meiosis I. Proc Natl Acad Sci USA 93:4730-4735.
4. Hashimoto N, Watanabe N, Furuta Y, Tamemoto H, Sagata N, Yokoyama M, Okazaki K, Nagayoshi M, Takeda N, Ikawa Y (1994): Parthenogenetic activation of oocytes in c-mos-deficient mice. Nature 370: 68-71.
5. Huo LJ, Fan HY, Zhong ZS, Chen DY, Schatten H, Sun QY (2004): Ubiquitin-proteasome pathway modulates mouse oocyte meiotic maturation and fertilization via regulation of MAPK cascade and cyclin B1 degradation. Mech Dev 121:1275-1287.
6. Ju JC, Tsay C, Ruan CW (2003): Alterations and reversibility in the chromatin cytoskeleton and development of pig oocytes treated with roscovitine. Mol Reprod Dev 64:482-491.
9. Knockaert M, Lenormand P, Gray N, Schultz P, Pouyssegur J, Meijer L (2002): p42/44 MAPKs are intracellular targets of the CDK inhibitor purvalanol. Oncogene 21: 6413-6424.
10. Krischek C, Meinecke B (2001): Roscovitine, a specific inhibitor of cyclin-dependent protein kinases, reversibly inhibits chromatin condensation during *in vitro* maturation of porcine oocytes. Zygote 9:309-316.
11. Kubelka M, Anger M, Kalous J, Schultz R, Motlik J (2002a): Chromosome condensation pig oocytes: lack of a requirement for either cdc2 kinase or MAP. Mol Reprod Dev 63:110-118.
12. Kubelka M, Anger M, Pavlok A, Kalous J, Schultz R, Motlik J (2002b): Activation of pig and cattle oocytes by butyrolactone I: morphological and biochemical study. Zygote 10:47-57.
13. Kubelka M, Motlik J, Schultz R, Pavlok A (2000): Butyrolactone I reversibly inhibits meiotic maturation of bovine oocytes without influencing chromosome condensation activity. Biol Reprod 62:292-302.
14. Liu Lin, Ju JC, Yang X (1998): Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. Biol Reprod 59: 537-545.
15. Maller JL, Schwab MS, Gross SD, Taieb FE, Roberts BT, Tunquist BJ (2002): The mechanism of CSF arrest in vertebrate oocytes. Mol Cell Endocrinol 187:173-178.
16. Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, Inagaki M, Delcros JG, Moulinoux JP (1997): Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2, and cdk5. Eur J Biochem 243:527-536.
17. Motlik J, Pavlok A, Kubelka M, Kalous J, Kalab P (1998): Interplay between cdc2 kinase and MAP kinase pathway during maturation of mammalian oocytes. Theriogenology 49:461-469.
18. Seong YS, Min C, Li L, Yang J, Kim Sy, Cao X, Kim K, Yuspa S, Chung HH, Lee K (2003): Characterization of a novel cyclin-dependent kinase 1 inhibitor, BMI-1026. Cancer Res 63:7384-7391.
19. Sobajima T, Aoki F, Kohmoto K (1993): Activation of mitogen-activated protein kinase during meiotic

maturation in mouse oocytes. J Reprod Fertil 97: 389-394.
20. Verlhac MH, Kubiak J, Clarke H, Maro B (1994): Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Development 120:1017-1025.
21. Verlhac MH, Kubiak J, Weber M, Geraud G, Colledge WH, Evans MJ, Maro B (1996): Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. Development 122:815-822.
22. Vigneron C, Perreau C, Dalbles-Tran R, Joly C, Humblot P, Uzbekova S, Mermillod P (2004): Protein synthesis and mRNA storage in cattle oocytes maintained under meiotic block by roscovitine inhibition of MPF activity. Mol Reprod Dev 69:457-467.

(Received: 2 March 2007/ Accepted: 20 April 2007)