A Prenylation Inhibitor (Sodium Phenylacetate) Differently Affects MCF-7 Cell Death when *ras* is Overexpressed, Partly Involving P42/44, JNK and P38 Kinase Activations

MÉLANIE DI BENEDETTO[1], MICHEL CRÉPIN[2], MICHEL KRAEMER[3] and OLIVIER OUDAR[3]

[1]*Laboratoire de Pharmaco-Oncologie Cellulaire, UMR-CNRS 7033 and*
[3]*EA 3410, Université Paris 13, 74 rue Marcel Cachin, 93017 Bobigny cedex;*
[2]*Laboratoire d'Hémostase, Endothélium et Angiogenèse (Unité INSERM 553),*
Hôpital Saint-Louis, 75010 Paris, France

Abstract. *Background: Sodium phenylacetate (NaPa) inhibits breast cancer cell proliferation decreasing prenylation of small G proteins including Ras. Materials and Methods: Aponecrosis induced by NaPa in MCF-7 and MCF-7ras breast cancer cells was evaluated by measuring Annexin V/PI labelling by flow cytometry. Specific inhibitors of p42/44 (PD 98059), p38 (SB 600125) and JNK (SP 202190) in association with NaPa were also tested. Mitogen-activated kinase (MAPK) activation was measured by immunoprecipitation. Results: NaPa induced cell death more efficiently (80%) in the MCF-7ras cells compared to the MCF-7 cells (60%). NaPa activated ERK 1/2 and its combination with PD 98059 decreased cell death in the MCF-7ras cells in contrast to the MCF-7 cells. Combination of NaPa with specific inhibitors of both JNK and p38 kinases also partly decreased MCF-7ras cell death. Conclusion: NaPa induced cell death differently when ras was overexpressed in breast cancer cells, partly involving p42/44, JNK and p38 pathways.*

Sodium phenylacetate (NaPa), a physiological metabolite of phenylalanine, is normally found in human plasma at micromolar concentrations. At higher concentrations, NaPa has been reported to induce the cytostasis and the reversion of malignant phenotype of different cancer cells by inhibiting prenylation of small G proteins such as Ras (1-5). Furthermore, NaPa has been shown to increase, in a synergistic manner, the effect of other antiproliferative agents (6-10). For example, NaPa potentiated the antitumor activity of tamoxifen, increasing apoptosis by BCL-2 down-regulation in MCF-7ras breast cancer cells (11). We have observed that NaPa alone induced MCF-7ras cell death associated with new morphological characteristics of both apoptosis and necrosis chimerically termed aponecrosis (12). NaPa has also been used in phase I and II clinical trials in patients with malignant tumors showing that the large drug doses required to reach a therapeutic plasma concentration (mM range) are well-tolerated with dose-limiting toxicity (13, 14). In addition, experiments with *ras*-transformed tumor cells have demonstrated that sensitivity to NaPa, associated with the inhibition of the prenylation of $p21^{ras}$, is due to an inhibition of the mevalonate pathway by this drug (1, 15).

The MCF-7 cell line transfected with the v-Ha-ras oncogene represents an estrogen-independent cellular model corresponding to some malignant breast tumors (16) and it does not require estrogen supplementation to induce a high incidence of tumors in nude mice in contrast to non-transfected cells (17). Furthermore, we have previously shown that MCF-7 cells transfected by *ras* present a different Ras mitogen-activated kinase (MAPK) pathway in response to fibroblast growth factor-2 (FGF-2), inducing DNA synthesis inhibition in contrast to non-transfected MCF-7 cells (18). These opposite effects are due to sustained activation of p42/44 MAPK (ERK 1/2) proteins in MCF-7ras cells whereas activation is transient in MCF-7 cells. Different functional signalling networks have been demonstrated when *ras* was overexpressed in response to mitogenic stimulation (19). Recently, integrated analysis of MCF-7 and MCF-7ras cells has demonstrated a different up-regulated pathway in the Ha-ras transformed cells (20).

Correspondence to: M. Di Benedetto, UMR-CNRS 7033, INSERM U553, 1 avenue Claude Vellefaux, 75010 Paris Cedex, France.Tel: +33 1 48 38 77 23, Fax: +33 1 48 38 88 65, e-mail: dibenedetto22@yahoo.fr

Key Words: Aponecrosis, mitogen-activated protein kinase, prenylation inhibitor, *ras*, NaPa.

Small G proteins such as Ras are the up-stream elements of MAPK pathways and considerable attention has been focused on the role of these signal transduction pathways in the regulation of cell survival. Among the three major MAPK pathways (p42/44 MAPK, JNK and p38), even if apoptosis is most often associated with JNK (c-Jun-N-terminal kinase) and p38 kinase activations, recent studies have demonstrated that p42/44 MAPK (ERK) activation can also be induced by noxious stimuli (21-24). NaPa has also been shown to decrease the activation of the $P21^{ras}$ target p42 MAPK/ERK2 (downstream element of Raf-1 and MEK1/2) in prostate cancer cells (25). Investigations of the functional roles of MAPK pathways in cell death, as well as other biological processes, have been greatly facilitated by the development of pharmacological inhibitors (26-28). Furthermore, to our knowledge the mechanism by which NaPa or prenylation inhibitors causes apoptosis, is still under investigation. Herein, the cell death induced by NaPa in MCF-7 and MCF-7ras cells has been compared and the involvement of MAPK pathways has been investigated using MAPK inhibitors.

Materials and Methods

Cell culture. The MCF-7 cells were purchased from American Tissue Culture Collection (ATCC, Rockville, MA, USA). The human breast cancer MCF-7ras cells, derived from pleural effusion MCF-7 cells and transfected with v-Ha-ras, were kindly provided by Dr C. Sommers (Georgetown University, Washington, DC, USA). The cells were all routinely grown in DMEM supplemented with L-glutamine 2 mM, penicillin 50 IU ml^{-1}, streptomycin 50 μg ml-1, and fetal calf serum (FCS) 10% (Life Technologies, Inc., Gaithersburg, MD, USA) at 37˚C in a 5% CO_2 humidified atmosphere.

Evaluation of cell death by flow cytometry. The MCF-7 or MCF-7ras ($1x10^5$) cells in 2% FCS/DMEM were treated for 72 h with or without 40 mM of NaPa, (conditions previously demonstrated to induce a significant amount of cell death (9) added with or without MAPK inhibitors PD98059 (10 μM,) (Sigma, Saint Louis, MO, USA), SB600125 (15 μM) (Biomol Res Lab, PA, USA) or SP202190 (15 μM) (Biomol Res Lab, PA, USA). After the different treatments, the cells were harvested, centrifuged and washed with annexin buffer (Boerhinger) as previously described (29). Briefly, to reveal a phosphatidylserine translocation specific to apoptosis stage, the cells were incubated with a FITC-labeled Annexin V (Boerhinger) (0.5 μg/ml) and PI (2.5 μg/ml) for 10 min in the dark. Then, the cells were analyzed by flow cytometry using single laser emitting exitation.

The propidium iodide (PI) enters into the cells during the ultimate stage of apoptosis or predominantly during the first stage of necrosis when damage of the cell membrane has occurred. Forward scatter (FSC) represents the size of the cells. The decline of cell size or damage to the plasma membranes induced by cell death can also be visualized by a reduction of the cell distribution in the FSC versus an increase in the side scatter (SSC) representing cell granulation.

The Annexin-V-positive/PI negative (Ann^+/PI^-) population corresponds to cells in early apoptotic phase and the Annexin-V-positive/PI positive ($Ann\text{-}V^+/PI^+$) one to cells in late apoptosis phase and/or necrosis. The latter population can also be revealed by trypan blue staining as previously described (29).

Measurement of MAPK activation. The cells ($1x10^6$) were grown in complete medium for 1 day and then starved for 24 h in serum-free medium prior to the incubation with 2% FCS/DMEM with or without NaPa 40 mM. Since the induction of MAPK signalling has been demonstrated to be a quick event in cell proliferation (18), the cells were treated with NaPa for 1 h. The PD 98059 (10 μM), SB600125 or SP202190 (15 μM) was added 1 h before the incubation with 2% FCS/DMEM with or without NaPa (40 mM). The cells were then washed twice with cold PBS containing 1 mM of Na_3Vo_4 and lysed in 1 ml lysis buffer 1x containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3Vo4 and 1 μg/ml Leupeptin according to the p44/42 MAP Kinase assay kit manufacturer recommendations (Cell Signalling Technology Inc, Beverly, MA, USA). The lysates were clarified by centrifugation at 14,000 xg for 10 min at 4˚C and 200 μl of the supernatant (total protein) were incubated with immobilized phospho-p42/44 MAPK, JNK or P38 kinase monoclonal antibody (Cell Signaling Technology Inc, Beverly, MA, USA) overnight at 4˚C. The immobilized phosphorylated proteins were then washed twice with kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na3Vo4, 10 mM $MgCl_2$). The kinase assays were performed by incubating the phosphorylated proteins in 50 μl kinase buffer containing 200 μM ATP and 2 μg Elk-1, AFT-2 or C-Jun fusion protein for 30 min at 30˚C. Ser 383 of Elk-1 is a major phosphorylation site by p42/44 MAPK and is required for Elk-1 dependent transcriptional activity (30). The p38 protein phosphorylate the AFT-2 transcriptional factor at the Thr 69 and Thr 71 sites (31). JNK binds to the amino-terminal region of c-Jun and phosphorylate c-jun at Ser 63/73 (32). The reaction was stopped by the addition of the SDS sample buffer and boiled samples were analysed by 10% SDS/PAGE and nitrocellulose membrane electrotransfer. After blocking the membranes with 5% bovine serum albumin (BSA) in 10 mM Tris pH 7.5, 150 mM NaCl and 0.1% Tween, they were incubated for 1 h with phospho-Elk1, AFT-2 and C-Jun polyclonal antibody (Cell Signaling Technology) diluted 1:1000, followed by incubation with horseradish-conjugated anti-rabbit IgG antibody (1:3000) and the bands were visualised using an enhanced chemiluminescence reagent system (Amersham pharmacia biotech, Buckinghamshire, UK). Also, the membranes were incubated with an anti-ERK1/2, anti-JNK or anti-p38 monoclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for 1 h diluted 1:1000, followed by incubation with horseradish-peroxidase-conjugated anti-rabbit IgG antibody (1:10000) and the bands were visualised as described above

Statistical analysis. Multiple statistical comparisons were performed using ANOVA in a multivariable linear model. Some statistical comparisons were conducted using the student *t*-test. $P<0.05$ was considered statistically significant.

Results

Effects of the p42/44 MAPK inhibitor (PD 98059) in combination with NaPa on MCF-7 or MCF-7ras cell death. NaPa (40 mM) induced MCF-7 cell death after 72 h of

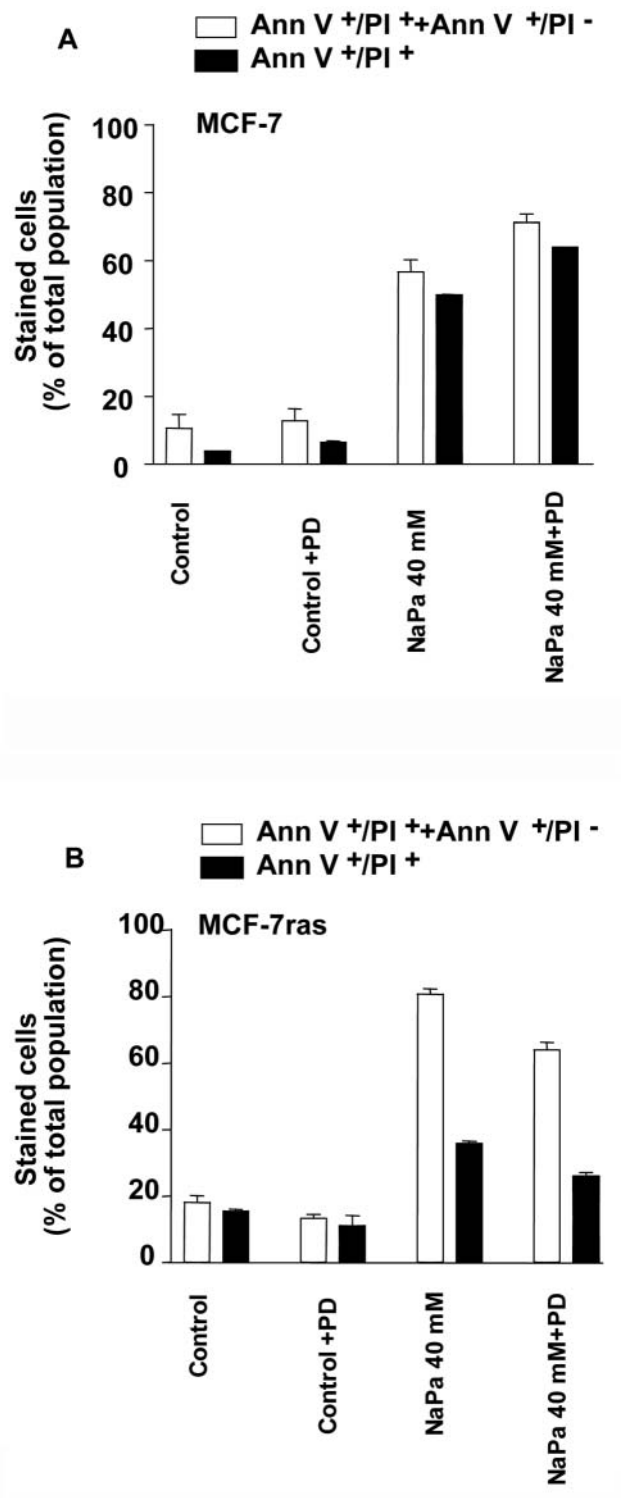

Figure 1. *Effect of NaPa alone or in combination with PD 98059 on MCF-7 and MCF-7ras cell death. MCF7 (A) or MCF-7ras cells (B) were incubated for 72 h in the presence or absence of NaPa (40 mM) alone or in combination with PD 98059 (10 μM). After staining with FITC Annexin-V (Ann-V) and propidium iodide (PI), the cells were analysed by flow cytometry. Ann-V^+/PI^-: early apoptosis or Ann-V^+/PI^+: late apoptosis and/or necrosis Mean (±S.D.) of three independent experiments.*

treatment (Figure 1A). We observed almost 60% of the cells were Annexin-V stained, approximatively 50% Ann-V^+/PI^+ (late apoptosis and/or necrosis) and 10 % Ann-V^+/PI^- (of cells in early phase apoptosis). The addition of the p42/44 MAPK inhibitor, PD 98059 (10 μM) did not significantly affect the viability of the untreated cells ($p=0.31$) while with the NaPa treated cells it slightly increased the percentage of cell death (70%). This slight enhancement was correlated with an increase in the proportion of cells in late apoptosis and/or necrosis (Ann-V^+/PI^+) to 65% whereas the cell population in the early stage of apoptosis (Ann-V^+/PI^-) remained unchanged (about 10%).

The percentage of cell death in the MCF-7ras cells was higher than in the MCF-7 cells (80% of Annexin-V positive cells, Figure 1B) and the population in the early stage of apoptosis was increased (44%). In contrast to MCF-7 cells, the combination of 10 μM of PD 98059 with 40 mM NaPa induced a reduction of MCF-7ras cell death in both early and late apoptosis phases ($p=0.0006$).

NaPa modulation of p42/44 MAPK activation. After serum-starvation the addition of 2% FCS/DMEM induced activation of p42/44 MAPK in the MCF-7 cells (Figure 2A, lane 1, top panel). As shown by the bottom panel, the addition of NaPa or PD 98059 did not influence the level of p42/44 protein expression. The addition of non-apoptotic concentrations of NaPa (5 mM and 20 mM) (9) inhibited the MAPK activation (lanes 2 and 3, respectively). However, NaPa at 40 mM induced an increase of the MAPK activation to the control level. The addition of the p42/44 MAPK inhibitor, PD 98059 at 10 μM effectively inhibited the activation of the MAPK pathway (lanes 5 to 8).

In the case of the MCF-7ras cells, the NaPa effect on the p42/44 pathway was different since the lower concentrations of NaPa (5 and 20 mM) did not significantly inhibit p42/44 MAPK activation (Figure 2B, top panel, lane 2 and 3). The higher NaPa concentration (40 mM, lane 4), increased p42/44 MAPK activation above that in the MCF-7ras control cells (lane 1). The addition of the PD 98059 inhibited the activation of MAPK (lanes 6 to 8). In all cases, the treatments with PD 98059 and NaPa did not influence the synthesis of the p42/44 MAPK protein (bottom panel).

Effect of the p38 MAPK inhibitor (SB 600125) in combination with NaPa on MCF-7 cell and MCF-ras cell death. The addition of SB 600125 to the NaPa increased MCF-7 cell death from 60% to 95% (Figure 3A). The p38 inhibitor increased the amount of cells in the early as well as in the late stage of apoptosis (10 to 20% and 50 to 75%, respectively). In the MCF-7ras cells (Figure 3B), the combination of SB 600125 with NaPa induced a decrease in the proportion of cells undergoing apoptosis from 80 to 50%. A reduction about 10%

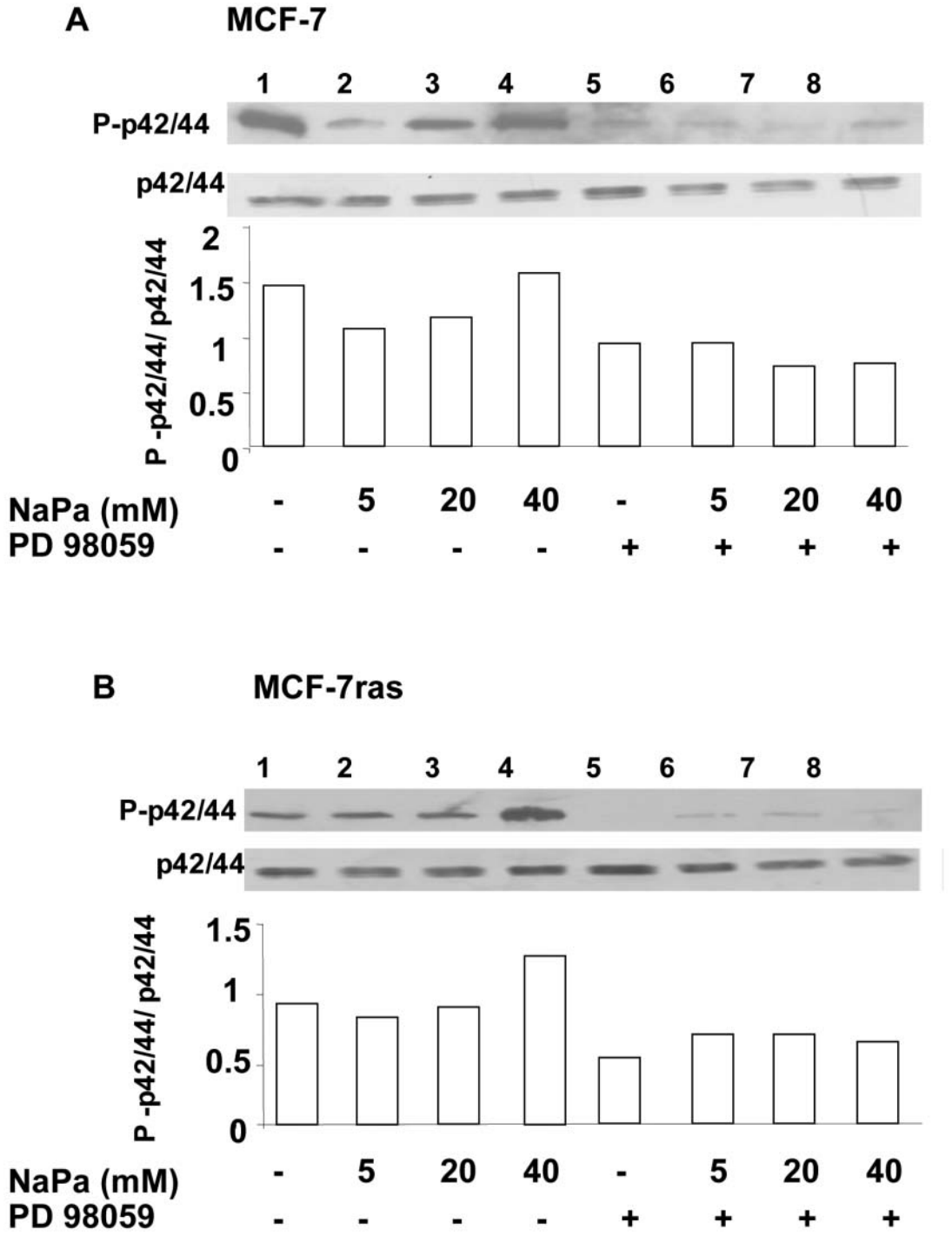

Figure 2. *Effect of NaPa on p42/44 MAPK activation in MCF-7 (A) and MCF-7ras (B) cells. Cells (1x10^6) were treated with NaPa alone (5, 20, 40 mM, lanes 2-4) or in combination with PD 98059 (10 μM, lanes 6-8) for 72 h. MAPK activity was measured by Elk-1 phosphorylation (top panel) and p42/44 protein expression (bottom panel). Bar graphs correspond to analysis of the most representative of at least two experiments performed separately.*

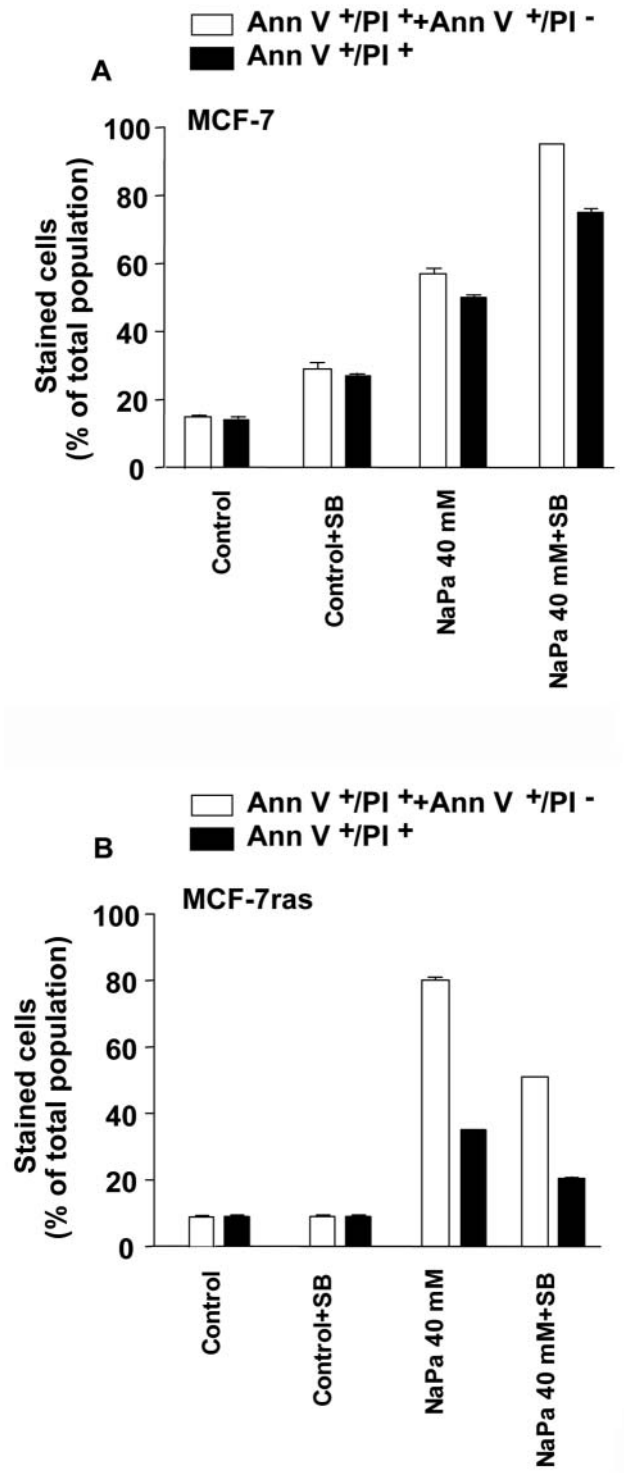

Figure 3. *Effect of NaPa alone or in combination with SB 600125 on MCF-7 and MCF-7ras cell death. MCF-7 cells (A) or MCF-7ras cells (B) were incubated for 72 h in the presence of NaPa (40 mM) alone or in combination with SB600125 (15 μM). After staining with FITC Annexin-V (Ann-V) and propidium iodide (PI), the cells were analysed by flow cytometry. Ann-V$^+$/PI$^-$: early apoptosis Ann-V$^+$/PI$^+$: late apoptosis and/or necrosis Mean (±S.D.) of three independent experiments.*

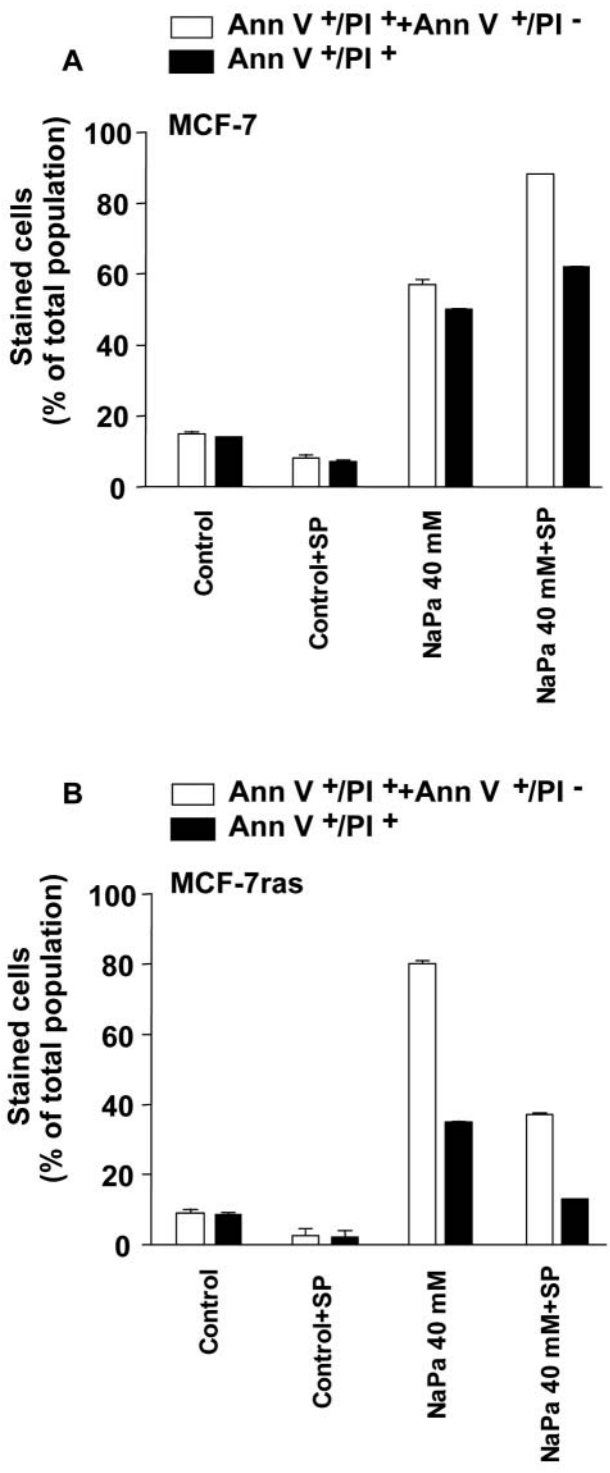

Figure 4. *Effect of NaPa alone or in combination with SP 202190 on MCF-7 and MCF-7ras cell death. MCF-7 (A) or MCF-7ras cells (B) were incubated for 72 h in the presence or absence of NaPa (40 mM) alone or in combination with SP202190 (15 μM). Cells were stained with FITC Annexin-V (Ann-V) and propidium iodide (PI) and analysed by cytometry. Ann-V$^+$/PI$^-$: early apoptosis Ann-V$^+$/PI$^+$: late apoptosis and/or necrosis Mean (±S.D.) of three independent experiments.*

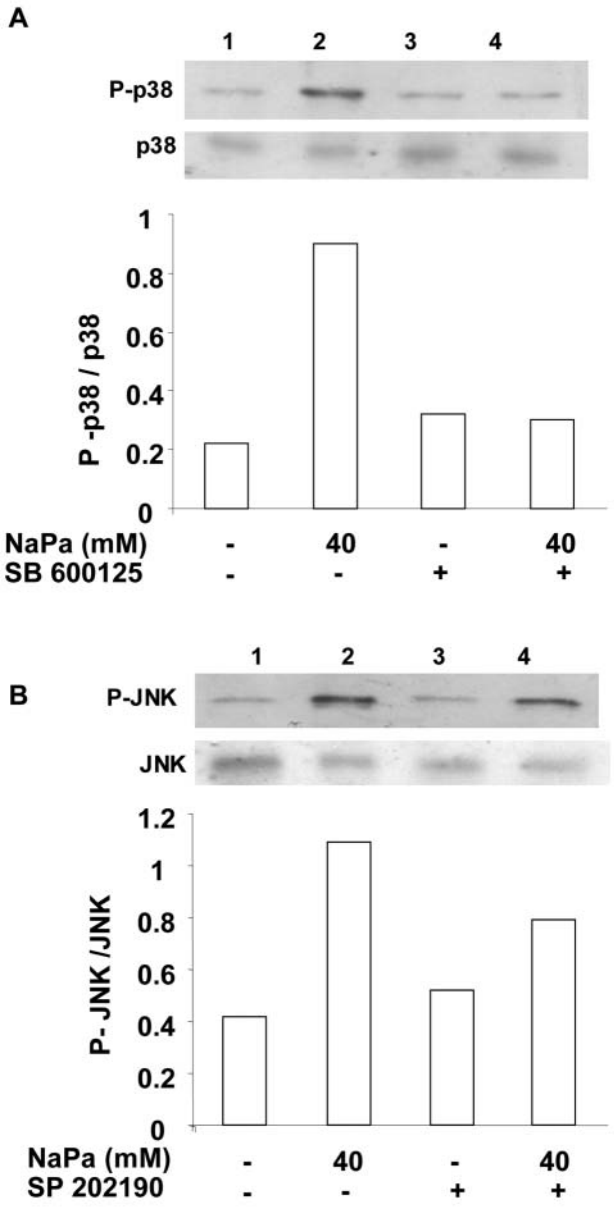

Figure 5. *Effect of NaPa on p38 and JNK activation in MCF-7ras cells. Cells (1x10^6) were treated with NaPa alone at 40 mM (lane 2) or in combination with inhibitor at 15 μM (lane 4). (A) Effect of NaPa on p38 activation, measured by AFT-2 phosphorylation measurement after immunoprecipitation of p38 (top panel), and p38 protein expression (bottom panel). (B) Effect of NaPa on JNK activation, measured by C-Jun phosphorylation (top panel) and JNK protein expression (bottom panel). Bar graphs correspond to the analysis of the most representative of at least two experiments performed separately.*

of cells in late apoptosis and about 20% of cells in early apoptosis was observed. The addition of the p38 inhibitor alone did not significantly affect the viability of either the MCF-7 (p=0.109) or the MCF-7ras cells (p=0.211).

Effect of the JNK MAPK inhibitor SP 202190 in combination with NaPa on MCF-7 and MCF-7ras cell death. The addition of the JNK inhibitor SP 202190 alone did not affect the viability of the MCF-7 or the MCF-7ras cells (Figure 4). The addition of SP 202190 to the NaPa increased the MCF-7 cell death (Figure 4A) in the same manner observed with the use of the p38 inhibitor. In contrast, inhibition of JNK activation in the MCF-7ras cells treated with NaPa induced a reduction of cells in both early and late stage of apoptosis (Figure 4B). In addition, SP 202190 in combination with NaPa 40 mM induced a decrease of 20% of cells in late apoptosis as compared to the effect of NaPa alone. A similar reduction was observed for the cells in early phase apoptosis.

Activation of JNK/p38 pathways induced by NaPa. As observed in the case of p42/44 protein activation, the addition of NaPa at 40 mM induced an increase in the ratio of activated p38/ total p38 proteins (Figure 5A). The NaPa increased about 4-fold the p38 substrate (AFT-2). The use of the inhibitor SB 600125 at 15 μM completely reversed the activation induced by NaPa. In the same manner, the addition of NaPa induced a 3-fold higher level of C-Jun phosphorylated protein as compared to control (Figure 5B). The addition of 15 μM of SP 600125 partially inhibited the JNK activation induced by NaPa.

Discussion

In the present study NaPa induced breast cancer cell death through the activation of the MAPK pathway when *ras* was overexpressed. NaPa induced activation of p42/44 MAPK in the MCF-7ras cells and the addition of PD 98059 in part reduced the cell death. In contrast, in the non-transformed cells (MCF-7), the use of inhibitors did not reverse apoptosis but increased it, suggesting an additive effect of p42/44 MAPK inhibition with others pathways. PD 98059 alone had no effect on the MCF-7 or the MCF-7ras cell viability. This was in agreement with other studies which showed that the disruption of this pathway by MAPK inhibitors was not always sufficient to induce apoptosis. It has also been demonstrated that the interruption of the MAPK cascade by the pharmalogical agents or other means, could potentate or reduce the apoptosis of cells exposed to other environmental stresses (21, 23, 33-36). NaPa (40 mM) induced MCF-7ras cell death (80%) with a majority of cells in early apoptosis (44%) after 72 h whereas in the case of the wild-type MCF-7 cells, the percentage of cell death was lower (60%) and only 10% of these cells were in early apoptosis stage. These results are in agreement with previous findings which demonstrated that NaPa or other prenylation inhibitors were more effective in inhibiting the proliferation of *ras*-transformed cells (1, 15). In each cell lines (MCF-7 or MCF-7ras), the different distribution of the

cell death populations (apoptotic and necrotic) was due to the time of treatment with NaPa since its effect was demonstrated to be time-dependent (9, 37).

In our experiments and according to Danesi *et al.* (25), NaPa (5 and 20 mM) inhibited the activation of ERK1/2 in MCF-7 cells at concentrations less than 40 mM. Those concentrations have been demonstrated to be implicated in the cell cycle arrest of both MCF-7 and MCF-7ras cells but did not affect their viability (7, 9). Samid *et al.*, have previously shown that these concentration-range of NaPa combined with PD 98059 increased cell cycle arrest by increasing $p21^{Cip1/Waf1}$ expression and by decreasing PPAR γ activation , a potential target of NaPa (4). Additionaly in contrast to the MCF-7 cells, NaPa at these concentrations did not induce differences in ERK1/2 activity in the MCF-7ras cells as compared with the control. This result could be explained, in part, by the overexpression of the Ras protein which led to increasing activation of the MAPK pathway in the MCF-7ras cells.

At a higher NaPa concentration, activation of the ERK1/2 protein was induced without affecting its synthesis in the MCF-7ras cells. In the MCF-7 cells, the activity of ERK 1/2 induced by NaPa (40 mM) after 1h of stimulation remained at the control level. Previous data have indicated that sustained activity of the MAPK activation (more than 1 h) in MCF-7 cells was associated with cell proliferation inhibition as well as DNA synthesis inhibition (18, 38). In our experiments, sustained activation was also observed over 2h in MCF-7ras cells (data not shown). The addition of p42/44 inhibitor although inhibiting activation, did not reverse MCF-7 apoptosis and this effect has also been observed recently (38). As mentioned above, inhibition of p42/44 MAPK activity may act additively with other pathways to increase MCF-7 cell death. The incomplete reversion of MCF-7ras cell death observed in the presence of MAPK cascade inhibitors, indicated that ERK 1/2 activation was partly involved in MCF7-ras cell death signalling. MCF-7ras Bcl-2 down-regulation previously observed in our laboratory after a long NaPa exposure (more than 4 days) (11) was probably the result of another undetermined NaPa down-stream mechanism involving protein expression inhibition as previously described (2). Furthermore, the death of the MCF-7ras cells induced by NaPa was also partly reduced by inhibition of both JNK and p38 activities. We have previously shown that the JNK pathway played a critical role in breast cancer cell proliferation (18). These results suggest that a direct or indirect undetermined mechanism implicates the three major MAPK protein activations (p42/44, JNK, p38) to induce *ras*-transformed cell death with the NaPa. Furthermore, a cross-talk pathway between p42/44 and p38 has been previously described although the mechanism is still not elucidated (27). In contrast, our results showed that the activation of these two pathways was not involved in the MCF-7 non-transformed cells, since inhibitors did not reduce cell apoptosis but increased as shown by the ERK1/2 inhibitor. As mentioned above, in the MAPK ERK1/2 pathway, the inhibition of p38/JNK activation could be associated with other pathways to induce MCF-7 cell death. The higher proportion of MCF-7 cell death increase with the p38 and JNK inhibitions in contrast to ERK1/2 inhibition (35% versus 10%, respectively) should be noted. These results could indicate the importance of inhibiting p38/JNK as compared to ERK1/2 activity to induce MCF-7 cell death and are in agreement with previous results that have shown the essential role of the MAP kinase pathway signalling in mammary epithelial cell survival (33). In addition, these results were supported by a recent report showing different pathways of cell death in MCF-7 as compared to MCF-7ras cells (20).

In conclusion, ERK, JNK and p38 are, in part, involved in MAPK activation in *ras*-transformed cell death induced by prenylation inhibitor, partly explaining the vulnerability of *ras*-transformed cells to NaPa and indicating that the effect of anchored Ras targeting drugs (such as NaPa) in combination with MAPK inhibitors depends on the level of *ras* expression.

Acknowledgements

This work was supported by a grant from the "Association pour la Recherche contre le Cancer (A.R.C)" and ''La Ligue Nationale contre le Cancer". We thank Odile Sainte-Catherine and Caroline Mangin for their excellent technical assistance.

References

1 Mo H and Elson CE: Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. Exp Biol Med *229*: 567-585, 2004.

2 Li Kn, Parikh S, Shu Q, Jung HL, Chow CW, Perlaky L, Leung HC, Su J, Blaney S and Lau CC: Phenylbutyrate and phenylacetate induce differentiation and inhibit proliferation of human medulloblastoma. Clin Cancer Res *10*: 1150-1159, 2004.

3 Ahmed F, Adsule S, Ali AS, Barnerjee S, Ali S, Kulkarni S, Padhye S and Sarkar FH: A novel copper complex of 3-benzoyl-alpha methyl benzene acetic acid with antitumor activity mediated *via* cyclooxygenase pathway. Int J Cancer *120*: 734-742, 2007.

4 Samid D, Wells M, Greene ME, Shen W, Palmer CN and Thibault A: Peroxisome proliferator-activated receptor gamma as a novel target in cancer therapy: binding and activation by an aromatic fatty acid with clinical antitumor activity. Clin Cancer Res *3*: 933-941, 2000.

5 Witzig TE, Timm M, Stenson M, Svingen PA and Kaufmann SH: Induction of apoptosis in malignant B cells by phenylbutyrate or phenylacetate in combination with chemotherapeutic agents. Clin Cancer Res *6*: 681-692, 2000.

6 Ferrandina G, Filippini P, ferlini C, Maggiano N, Stler AB, Fruscella E, Mozetti S, Mancuso S, Freedman RS, Scambia G and Ranelletti FO: Growth inhibitory effects and radiosensitization induced by fatty aromatic acids on human cervical cancer cells. Oncol Res *12*: 429-440, 2000.

7 Thibout D, Di Benedetto M, Kraemer M, Sainte-Catherine O, Derbin C and Crepin M: Sodium phenylacetate modulates the synthesis of autocrine and paracrine growth factors secreted by breast cancer cell lines. Anticancer Res *18*: 2657-2662, 1998.

8 Prasanna P, Thibault A, Liu L and Samid D: Lipid metabolism as a target for brain cancer therapy: synergistic activity of lovastatin and sodium phenylacetate and phenylbutyrate. Clin Cancer Res *2*: 865-872, 1996.

9 Di Benedetto M, Kourbali Y, Starzec A, Vassy R, Jozefonvicz J, Crepin M and Kraemer M: Sodium phenylacetate enhances the inhibitory effect of dextran derivative on breast cancer cell growth both *in vitro* and in nude mice. Br J Cancer *85*: 917-923, 2001.

10 Wei MX, Liu JM, Gadal F, Yi P, Liu J and Crepin M: Sodium phenylacetate (NaPa) improves the TAM effect on glioblastoma experimental tumors by inducing cell growth arrest and apoptosis. Anti cancer Res *27*: 953-958, 2007.

11 Adam L, Crepin M and Israel L: Tumor growth inhibition, apoptosis, and Bcl-2 down-regulation of MCF-7ras by sodium phenylacetate and tamoxifen combination. Cancer Res *57*: 1023-1029, 1997.

12 Formigli L, Papucci L, Tani A, Schiavone N, Tempestini A, Orlandini GE, Capaccioli S and Orlandini SZ: Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J Cell Physiol *182*: 41-49, 2000.

13 Chang SM, Kuhn JG, Ian Robins H, Clifford Schold S, Spence AM, Berger MS, Mehta MP, Pollack I, Gilbert M and Prados MD: A study of a different dose-intense infusion schedule of phenylacetate in patients with recurrent primary brain tumors consortium report. Invest New Drug *21*: 429-433, 2003.

14 Thompson P, Balis F, Serabe BM, Berg S, Adamson P, Klenke R, Aiken A, Packer R, Murry DJ, Jakacki R and Blaney SM: Pharmacokinetics of phenylacetate administered as a 30-min infusion in children with refractory cancer. Cancer Chemother Pharmacol *52*: 417-423, 2003.

15 Shack S, Chen LC, Miller AC, Danesi R and Samid D: Increased susceptibility of *ras*-transformed cells to phenylacetate is associated with inhibition of p21ras isoprenylation and phenotypic reversion. Int J Cancer *63*: 124-129, 1995.

16 Spandidos DA and Agnantis NJ: Human malignant tumors of the breast as compared to their respective normal tissue have elevated expression of the Harvey *ras* oncogene. Anticancer Res *4*: 269-272, 1984.

17 Sommers CL, Papageorge A, Wilding G and Gelmann EP: Growth properties and tumorigenesis of MCF-7 cells transfected with isogenic mutants of ras^H. Cancer Res *50*: 67-71, 1990.

18 Liu JF, Issad T, Chevet E, Ledoux D, Courty J, Caruelle JP, Barritault D, Crepin M and Bertin B: Fibroblast growth factor-2 has opposite effects on human breast cancer MCF-7 cell growth depending on the activation level of mitogen-activated protein kinase pathway. Eur J Biochem *258*: 271-276, 1998.

19 Liu JF, Chevet E, Kebache S, Lemaitre G, Barritault D, Larose L and Crepin M: Functional Rac-1 and Nck signalling networks are required for FGF-2 induced DNA synthesis in MCF-7 cells. Oncogene *18*: 6425-6433, 1999.

20 Gadal F, Bosic C, Pillot-Brochet C, Malinge S, Wagner S, Le Cam A, Buffat L, Crepin M and Iris F: Integrated transcriptome analysis of the cellular mechanisms associated with Ha-ras-dependent malignant transformation of the human breast epithelial MCF7 cell line. Nucleic Acids Res *31*: 5789-5804, 2003.

21 Dai Y, Yu C, Singh V, Tang L, Wang Z, McInistry R, Dent P and Grant S: Pharmacological inhibitors of the mitogen-activated protein kinase (MAPK) kinase/MAPK cascade interact synergically with UCN-01 to induce mitochondrial dysfunction and apoptosis in human leukaemia cells. Cancer Res *6*: 5106-5115, 2001.

22 Seidman R, Gitelman I, Sagi O, Band Horwitz S and Wolfson M: The role of ERK1/2 and p38 MAP-Kinase pathways in taxol-induced apoptosis in human ovarian carcinoma cells. Exp Cancer Cell Res *268*: 84-92, 2001.

23 Grant ER, Errico MA, Emanuel SL, Benjamin D, McMillian MK, Wadsworth SA, Zivn RA and Zhong Z: Protection against glutamate toxicity through inhibition of the p44/42 mitogen-activated protein kinase pathway in neuronally differentiated P19 cells. Biochem Pharmacol *62*: 283-296, 2001.

24 Amran D, Sancho P, Fernandez C, Esteban D, Ramos AM, de Blas E, Gomez M, Pallacios MA and Aller P: Pharmalogical inhibitors of extracellular signal-regulated protein kinases attenuate the apoptotic action of cisplatin in human myeloid leukaemia cells *via* glutathione-independent reduction in intracellular drug accumulation. Biochim Biophys acta *1743*: 269-279, 2005.

25 Danesi R, Nardini D, Basolo F, Del Tacca M, Samid D and Myers CE: Phenylacetate inhibits protein isoprenylation and growth of the androgen-independent LCCaP prostate cancer cells transfected with the T24 Ha-ras oncogene. Mol Pharmacol *49*: 972-979, 1996.

26 Dudley DT, Pang L, Decker SJ, Bridges AJ and Saltiel AR: A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA *92*: 7686-7689, 1995.

27 Singh RP, Dhawan P, Golden C, Kapoor GS and Mehta KD: One way cross-talk between p38 (MAPK) and p42/44 (MAPK): Inhibition of p38 (MAPK) induces low density lipoprotein receptor expression through activation of the p42/44 (MAPK) cascade. J Biol Chem *274*: 19593-19600, 1999.

28 Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Bhagwat SS, Manning AM and Anderson DW: SP 600125, an anthrapyrazolonze inhibitor of Jun, N-terminal kinase. Proc Natl Acad Sci *98*: 13681-13687, 2001.

29 Vermes I, Haanen C, Steffens-Naiken H and Reutelingsperger C: A novel assay for apoptosis. Flow cytometric detection of phoshatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Meth *184*: 39-51, 1995.

30 Gille H, Kortenjann M, Thomae O, Moomaw C, Slaughter C, Cobb MH and Shaw PE: ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. Embo J *14*: 951-962, 1995.

31 Van Dam H, Wilhelm D, Herr I, Steffen A, Herrlich P and Angel P: ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. Embo J *14*: 1798-1811, 1995.

32 Kyriakis JM and Avruch J: Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev *81*: 807-869, 2001.
33 Finlay D, Heavy L, Furlong F, O' Connell FC, Keon NK and Martin F: MAP Kinase pathway signalling is essential for extracellular matrix determined mammary epithelial cell survival. Cell Death Diff *7*: 302-313, 2000.
34 Golding SE, Rosenberg E, Neill S, Dent P, Povirk LF and Valerie K: Extracellular signal-related kinase positively regulates ataxia, telangiectasia mutated, homologus recombination repair and the DNA damage response. Cancer Res *67*: 1046-4053, 2007.
35 Son MH, Kang KW, Lee CH and Kim SG: Potentiation of cadmium-induced toxicity by sulfur-amino acid deprivation through activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in conjunction with p38 kinase or c-jun *N*-terminal kinase (JNK) complete inhibition of the potentiated toxicity by U0126 an ERK 1/2 and p38 inhibitor. Biochem Pharmacol *62*: 1379-1390, 2001.
36 Han B, Hua F, cao T, Dong H, Yang T, Yang Y, Pan H and Xu C: Requirement for ERK activity in sodium selenite-induced apoptosis of acute promyelocytic leukaemia-derived NB4 cells. J Biol Chem *40*: 196-204, 2007.
37 Di Benedetto M, Starzec A, Colombo BC, Briane D, Perret GY, Kraemer M and Crepin M: Aponecrotic, antiangiogenic and antiproliferative effects of a novel dextran derivative on breast cancer growth both *in vitro* and *in vivo*. Br J Pharmacol *135*: 1859-1871, 2002.
38 Zeng A, Kallio A and Harkonen P: Tamoxifen-induced rapid death of MCF-7 breast cancer cells is mediated *via* extracellular signal regulated kinase signalling can be abrogated by estrogen. Endocrinol *148*: 2764-2777, 2007.

Received September 18, 2007
Revised November 14, 2007
Accepted December 11, 2007