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# Cycloprodigiosin hydrocloride suppresses tumor necrosis factor (TNF) α-induced transcriptional activation by NF-κB

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**Abstract Cycloprodigiosin hydrochloride (cPrG·HCl) obtained from a marine bacterium *Pseudoalteromonas denitrificans* induces apoptotic cell death in various cancerous cell lines. cPrG·HCl alone caused a little cytotoxicity in HeLa cells, but it enhanced the apoptotic process progressively when co-administered with tumor necrosis factor (TNF)α. Here we studied the effect of cPrG·HCl on TNFα-induced activation of the transcription factor nuclear factor κB (NF-κB). Luciferase gene reporter assays revealed that cPrG·HCl potently suppressed the TNFα- and the phorbol myristate acetate-induced activation of NF-κB. The suppression occurred in the presence of imidazole, indicating that it was not related to the intracellular acidification resulting from the intrinsic $H^+/Cl^-$ symporter activity of cPrG·HCl. cPrG·HCl inhibited neither the TNFα-induced phosphorylation and degradation of inhibitor of nuclear factor-κB, nor the subsequent nuclear translocation and DNA binding of NF-κB. cPrG·HCl also suppressed NF-κB-enhanced gene expression induced by Rac1, Cdc42, MEKK1, inhibitor of nuclear factor-κα (IKKα), IKKβ, and a subunit of NF-κB, p65. These results indicate that cPrG·HCl suppresses NF-κB-dependent gene expression through the inhibition of transcriptional activation.** © 2000 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

*Key words:* Cycloprodigiosin; Nuclear factor κB; Tumor necrosis factor; IκB kinase; Apoptosis; Anti-tumor

## 1. Introduction

Tumor necrosis factor (TNF) α is a proinflammatory cytokine produced by many types of cells such as macrophages and monocytes. TNFα binds to the TNF receptors (TNFRs), and its signal is transduced through several TNFR-associated signal proteins including TNFR-associated death domain protein (TRADD), Fas-associated death domain protein (FADD), receptor-interacting protein (RIP) and TNFR-associated factor 2 (TRAF2). Among these, TRADD and FADD recruit caspase-8 which on activation initiates the apoptotic process and activation of downstream effector caspases, including caspase-3 [1–4]. Conversely, the signal from RIP and TRAF2 induces nuclear factor κB (NF-κB) activation, which is considered to have survival promoting effects on cells and counteract the cytotoxicity of TNFα [5–11].

NF-κB is a heterodimer transcription factor composed of p65 and p50, whose activation is induced by cytokines such as IL-1 and TNFα as well as proliferative agents such as phorbol myristate acetate (PMA) and bacterial lipopolysaccharide. Upon stimulation by these agents, the inhibitor of NF-κB (IκB)-kinases (IKKα and IKKβ) phosphorylate the inhibitory proteins (IκBs) associated with NF-κB in the cytoplasm. This is followed by degradation of the phosphorylated IκBs by the proteasome. Subsequently, NF-κB is translocated into the nucleus and binds to target DNA sequences to induce gene transcription [12,13].

Cycloprodigiosin hydrochloride (cPrG·HCl), a member of the prodigiosin family, is the red pigment produced by the marine bacterium *Pseudoalteromonas denitrificans* [14]. cPrG·HCl induces apoptosis in various cancer cell lines including rat and human hepatocellular cancer cells, promyelocytic leukemia cells (HL-60), as well as human breast cancer cells both in vitro and in vivo [15–17]. A similar observation has been reported recently in that prodigiosin from *Serratia marcescens* induces apoptosis in hematopoietic cancer cells without inducing any cytotoxicity in non-malignant cells [18]. Although the mechanism underlying this anti-cancer effect of the prodigiosin family is still unclear, it should be noted that cPrG·HCl, as well as other prodigiosins, acts as an $H^+/Cl^-$ symporter [19–22] which in turn may induce acidification of the cytosol, since $Cl^-$ ions are generally less abundant in the cytosol than in the extracellular milieu. Furthermore, although it is controversial [23], the intracellular acidification is thought to be a prerequisite for the apoptotic process. In fact, it has been demonstrated that cPrG·HCl induces intracellular acidification concomitantly with the induction of apoptosis in cancer cells [15,16]. In addition, cPrG·HCl-induced apoptosis is potently suppressed by a membrane permeable weak base imidazole which reverses the intracellular acidification [15,16]. Thus, it is most plausible that the apoptotic effect of cPrG·HCl in these cancer cells is caused by acidification of the cytosol. However, not all of the biological effects of cPrG·HCl are related to acidification of the cytosol, since cPrG·HCl induces the differentiation of HL-60 cells into monocytes, even in the presence of imidazole [16]. Thus, cPrG·HCl modulates cell physiology through intracellular pH modification and/or unknown molecular target(s).

cPrG·HCl and its related compounds can also act as immu-

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*Abbreviations:* cPrG·HCl, cycloprodigiosin hydrochloride; IκB, inhibitor of nuclear factor-κB; IKK, inhibitor of nuclear factor-κB kinase; NF-κB, nuclear factor κB; PMA, phorbol myristate acetate; TNF, tumor necrosis factor

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nosuppressants which are selective inhibitors of T cell proliferation [14]. Since cPrG·HCl-induced apoptosis in the human T cell derived Jurkat cell line, the apoptotic process is possibly involved in its immunosuppressive effect [14]. It is intriguing that a cPrG·HCl-related compound, PNU156804, suppresses the activation of both NF-κB and AP-1 in primary cultured human T cells [24]. Although the mechanism by which PNU156804 suppresses NF-κB activation is not clear, these results suggest that the suppression of NF-κB is involved in its immunosuppressive activity. This raises the question as to whether a prodigiosin family compound, cPrG·HCl, also modulates NF-κB activity in the cells. Here we examined the effect of cPrG·HCl on the signalling pathway of TNFα, and found that cPrG·HCl suppressed the gene expression mediated by NF-κB.

## 2. Materials and methods

### 2.1. Materials

The plasmids, pRKF-IKKα and pRKHA-IKKβ, have been described previously [25]. pRKF-IKKαEE and pRKHA-IKKβEE encode constitutively active mutants of IKKα, in which 176 and 180 serine residues in an activation loop are replaced by glutamic acid residues, and IKKβ, in which 177 and 181 serine residues are replaced by glutamic acid residues, respectively. A reporter plasmid pIG3Luc for NF-κB encodes triple NF-κB-binding site sequences (AGCTTCAGAGGGGACTTTCCGAGAGG) followed by the luciferase gene. A control plasmid, pBKRLuc, was obtained from Toyobo (Toyo B-Net Co., Japan). pcDNAF-MEKK1, pRKF-p65, pEFBOSHA-Rac1-DA, and pEFBOSHA-Cdc42-DA, are the expression plasmids for MEKK1, the NF-κB subunit p65, constitutively active Rac1 and Cdc42, respectively. Anti-p50 and anti-p65 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-IκBα antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.2. Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/ml kanamycin at 37°C in 5% $CO_2$ in air.

### 2.3. Assay for apoptotic processes

Cell viability was determined by dye exclusion assay using 0.1% trypan blue. DNA fragmentation was analyzed as described previously [14]. In the caspase-3 assay, cells were disrupted by freeze–thaw in an extraction buffer (50 mM Tris–HCl, pH 7.4, 1 mM EGTA, 5 mM EDTA, 5 mM $MgCl_2$, 1 mM APMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM DTT). Then the suspension was centrifuged at 15 000 rpm for 20 min, and the supernatants obtained were incubated at 37°C for 60 min in a reaction buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 10 mM EGTA) with 20 μM of the fluorogenic substrate DEVD-AFC (Peptide Institute, Inc., Japan). Cleavage of the substrates was quantified by measuring the fluorescence of 7-amino-4-trifluoromethyl coumarin released using a spectrofluorometer (Shimadzu RF5000) with excitation at 380 nm and emission at 460 nm.

### 2.4. Luciferase gene reporter assay and kinase assays of IKK

HeLa cells were transfected with the reporter plasmids described above using Lipofectin (Gibco BRL). After 24–48 h, the luciferase activity was determined using the luciferase assay system (Promega). The kinase activity of IKKα and IKKβ were analyzed by immune complex kinase assay as described previously using GST-IκBα (1–55) as a substrate [25].

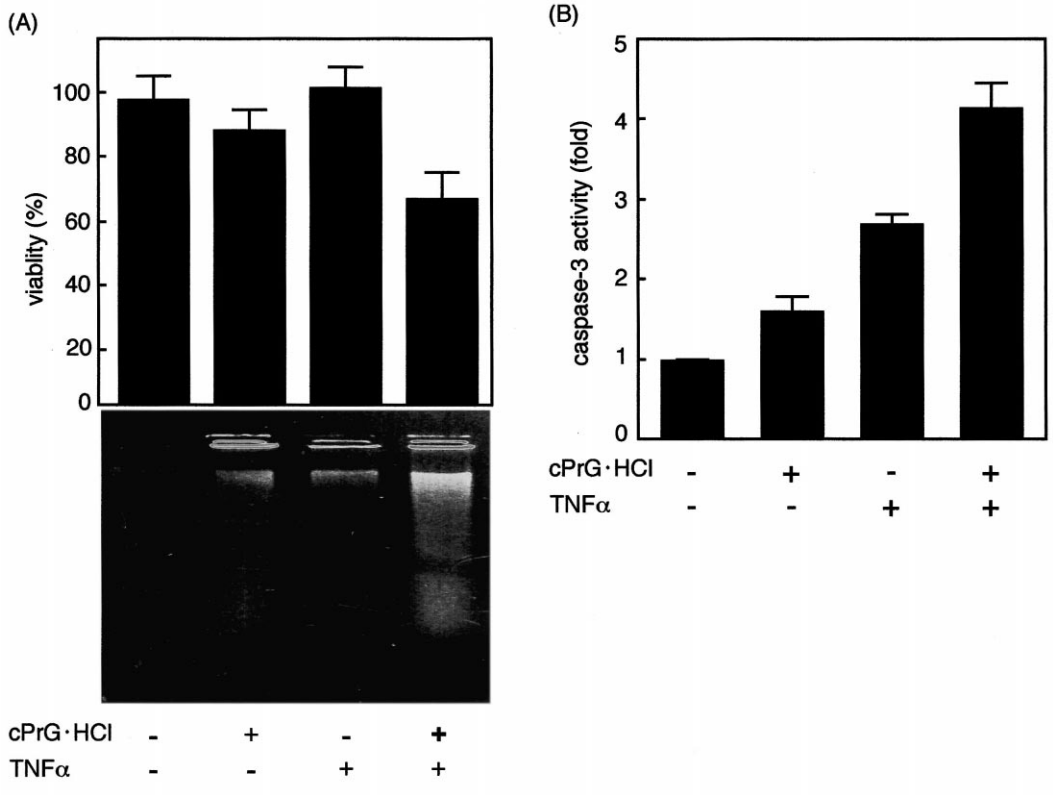


Fig. 1. cPrG·HCl induces apoptosis in TNFα-stimulated HeLa cells. A: (Upper panel) HeLa cells were cultured in the presence or absence of TNFα (20 ng/ml) with or without cPrG·HCl (1 μM) for 18 h. The viable cells were counted by trypan blue exclusion assay. (Lower panel) Soluble DNA was extracted from HeLa cells cultured in the presence or absence of TNFα (20 ng/ml) supplemented with or without cPrG·HCl (1 μM) for 24 h. Each soluble DNA sample (20 μg) was analyzed by electrophoresis on an agarose gel. B: HeLa cells were cultured in the presence or absence of TNFα (20 ng/ml) supplemented with or without cPrG·HCl (1 μM) for 8 h. Caspase-3 activity was estimated by using fluorogenic peptides.

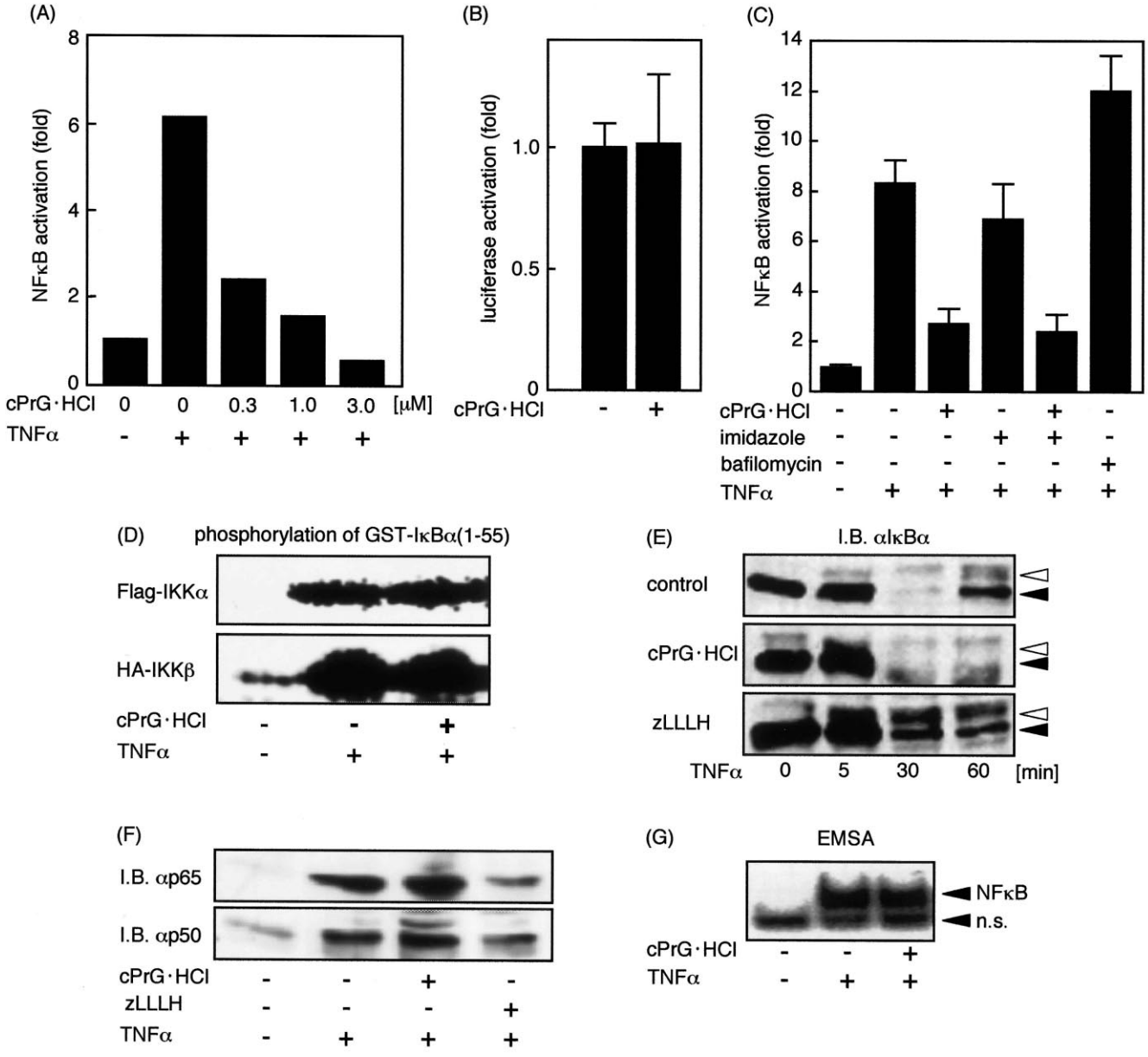


Fig. 2. cPrG·HCl suppresses NF-κB-dependent gene expression without suppressing TNFα-induced IκB degradation or the DNA binding of NF-κB. A: HeLa cells were transfected with pIG3Luc, which contained three NF-κB-binding sites upstream of the luciferase gene. After 24 h, the cells were treated with cPrG·HCl at various concentrations for 1 h before stimulation with TNFα (20 ng/ml). Following 4 h incubation with TNFα, NF-κB activity was measured by the luciferase assay. The luciferase activity was normalized by the protein content in each sample. The results are the mean of duplicate experiments. B: HeLa cells were transfected with pBKRLuc, which encoded the luciferase gene under the control of the CMV promoter. After 24 h, the cells were treated with or without cPrG·HCl (1 μM) for 5 h, and luciferase activity was measured. C: HeLa cells were transfected with pIG3Luc. After 24 h, the cells were treated with cPrG·HCl in the presence or absence of imidazole (10 mM) or bafilomycin (1 μM) for 1 h, and then were stimulated by TNFα (20 ng/ml) for 5 h. The results of the luciferase assay are presented as mean ± S.D. from three independent experiments. D: The cells were transfected with pRKF-IKKα or pRKHA-IKKβ. After 24 h, the cells were treated with cPrG·HCl (1 μM) for 1 h, and then were stimulated by TNFα (20 ng/ml) for 10 min. The transfected IKKα or IKKβ were recovered by immunoprecipitation, and the kinase activity was estimated using GST-IκBα (1–55) as a substrate. E: The cells were pretreated with cPrG·HCl (1 μM) for 1 h, and then were stimulated by TNFα (20 ng/ml). The phosphorylation and degradation of IκBα were analyzed by Western blotting using anti-IκB antibody. The black arrow heads indicate IκBα, and the white arrow heads indicate phosphorylated IκBα. F: Nuclear extracts were prepared from the cells treated with TNFα (20 ng/ml) for 30 min in the presence or absence of cPrG·HCl (1 μM) or zLLLH (10 μM). Nuclear translocation of p65 and p50 was analyzed by Western blotting. G: DNA-binding activity of NF-κB in the nuclear extract was analyzed by EMSA. n.s. means a non-specific binding.

*2.5. Electrophoretic mobility shift assay (EMSA)*

Nuclear extracts were prepared by the method of Schreiber, et al. [26]. Briefly, cells were solubilized with a buffer (10 mM HEPES–NaOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.4% NP-40, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT), and then centrifuged at 10 000 rpm for 15 min. The pellets were resuspended in a buffer (20 mM HEPES–NaOH, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 μg/ml antipain, 1 mM DTT), and then centrifuged at 15 000 rpm for 15 min. The supernatant was used as the nuclear extract. $^{32}$P-labeled DNA probes of the NF-κB-binding site (AGCTT-

CAGAGGGGACTTTCCGAGAGGTCGA) were prepared as described previously [27]. The nuclear extract (10 μg of protein) was incubated with the $^{32}$P-labeled probes (100 000 cpm) in 20 μl of buffer (20 mM HEPES–NaOH, pH 7.9, 5% glycerol, 1 mM EDTA, 100 μg/ml poly(dI-dC)) for 20 min at room temperature. The samples were electrophoresed on an 8% polyacrylamide gel and analyzed by an Imaging plate (Fuji Film).

*2.6. Western blot analysis*

Cells were solubilized with a buffer (20 mM Tris–HCl, pH 7.4, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 20 mM NaF, 1 mM APMSF, 50 u/ml aprotinin, 20 μg/ml pepstatin, 20 μg/ml leupeptin, 1 mM DTT, and 1% Triton X-100), and then centrifuged at 15 000 rpm for 20 min. The supernatants were used as the cell extracts. The cell extracts and the nuclear extracts were subjected to SDS–polyacrylamide gel electrophoresis, and transferred to an ECL membrane (Amersham Pharmacia). Western blot analysis was performed using a Western blotting detection system (Amersham-Pharmacia) according to the manufacturer's instructions.

## 3. Results

### *3.1. cPrG·HCl induces apoptosis in TNFα-stimulated HeLa cells*

We have reported previously that cPrG·HCl induces apoptosis in PMA-stimulated Jurkat cells as well as in various human cancer cell lines [14,15,17]. In HeLa cells, cPrG·HCl alone caused relatively little cytotoxicity, while the viability was reduced to less than 65% of control when cells were treated by cPrG·HCl together with TNFα (Fig. 1A). DNA fragmentation was observed in these co-stimulated cells and, consistent with this, cPrG·HCl activated caspase-3 in cells treated with TNFα (Fig. 1B). Thus, cPrG·HCl potentiates the TNFα-induced activation of caspase-3, and enhances the apoptotic process. Although treatment of HeLa cells with TNFα alone increased the activity of caspase-3, there was no loss in the cell viability (Fig. 1A,B).

### *3.2. cPrG·HCl suppresses TNFα- and PMA-induced activation of NF-κB without attenuating upstream cellular signalling pathways*

It has been shown that TNFα activates NF-κB, which in turn leads to the expression of certain genes, such as the inhibitor of apoptosis (IAP), to promote cell survival. Indeed, the suppression of NF-κB results in the potentiation of TNFα cytotoxicity [6,7,10,11]. This prompted us to study the effect of cPrG·HCl on activation of NF-κB activation. The cells were pre-treated with cPrG·HCl for 1 h to exhibit inhibitory effects on NF-κB activation and the cellular signalling of TNFα, if any. The luciferase gene reporter assay revealed that cPrG·HCl suppressed the gene expression dependent on TNFα-induced NF-κB activation in a dose-dependent manner (Fig. 2A). As a control, cPrG·HCl completely failed to affect the expression of the luciferase gene under the control of the CMV promoter in the reporter assay using a control vector, pBKRLuc (Fig. 2B). It was intriguing that cPrG·HCl also inhibited NF-κB transcription activity, even in the presence of the cell permeable weak base imidazole. This indicated that the suppression was not a consequence of the intracellular acidification due to the intrinsic $H^+/Cl^-$ symporter activity of cPrG·HCl (Fig. 2C). A vacuolar-type ATPase inhibitor, bafilomycin, which is also known to induce intracellular acidification, did not suppress NF-κB activation (Fig. 2C). Thus, the inhibitory effect of cPrG·HCl on NF-κB is not a result of intracellular acidification.

TNFα activates NF-κB through the activation of IKKα and IKKβ, phosphorylation and degradation of IκB, and nuclear translocation of NF-κB. To determine which step is inhibited by cPrG·HCl, we analyzed the effects of this compound on the TNFα signalling systems. When cells were stimulated with TNFα, both IKKα and IKKβ were markedly activated either in the presence or absence of cPrG·HCl, indicating that cPrG·HCl did not inhibit the signalling from the TNFr to IKKα and IKKβ (Fig. 2D). Consistent with the activation of IKKα and IKKβ, the phosphorylation of IκB was rapidly induced within 5 min in the presence or absence of cPrG·HCl as revealed by a mobility shift of electrophoresis (Fig. 2E). After 30 min, the phosphorylated IκB was degraded by proteasome, and this degradation was effectively suppressed by a proteasome inhibitor, zLLLH. In contrast, cPrG·HCl did not inhibit the degradation of the phosphorylated IκB. Although the degradation of IκB was unaffected by cPrG·HCl, it should be noted that the re-synthesis of IκB after 60 min of TNFα stimulation was potently suppressed by cPrG·HCl. This is consistent with the observation that the induction of the IκB gene by extracellular stimuli following IκB degradation is mediated by NF-κB-dependent gene expression. The suppression of the re-synthesis of IκB in the

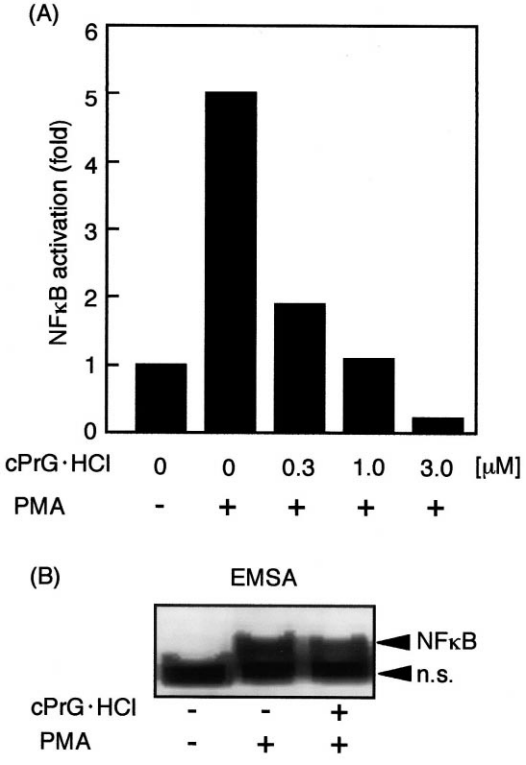


Fig. 3. cPrG·HCl suppresses PMA-induced NF-κB activation. A: HeLa cells were transfected with pIG3Luc. After 24 h, the cells were treated with cPrG·HCl at various concentrations for 1 h before stimulation with PMA (1 μM). Following 4 h incubation with PMA, NF-κB activity was measured by the luciferase assay. The luciferase activity was normalized by the protein content in each sample. B: Nuclear extracts were prepared from the cells treated with PMA for 30 min in the presence or absence of cPrG·HCl (1 μM). The nuclear translocation and the DNA-binding activity of NF-κB were analyzed by EMSA. n.s. means a non-specific binding.

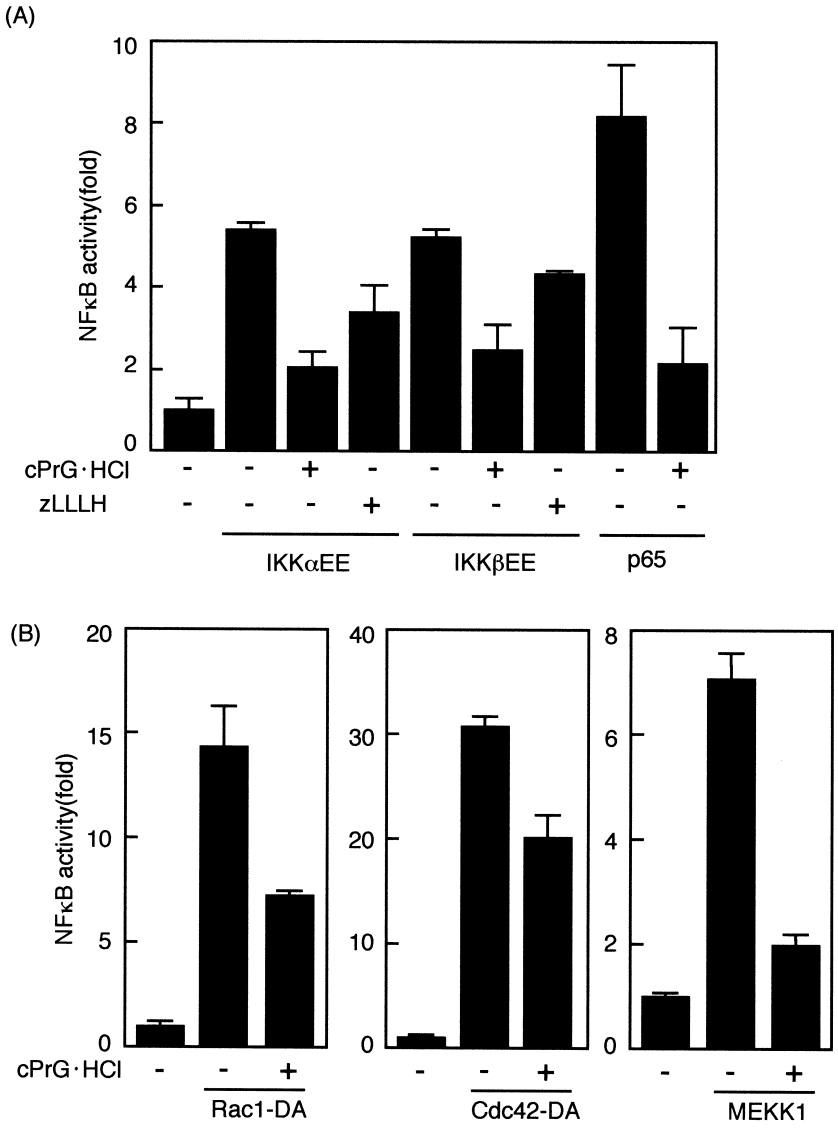


Fig. 4. cPrG·HCl suppresses IKKαEE-, IKKβEE-, p65-, Rac1-, Cdc42- and MEKK1-induced NF-κB transcriptional activity. HeLa cells were transfected with (A) pRKF-IKKαEE, pRKHA-IKKβEE, pRKF-p65, (B) pEFBOSHA-Rac1-DA, pEFBOSHA-Cdc42-DA, or pcDNAF-MEKK1 together with the reporter plasmid pIG3Luc and pBKRLuc. After 24 h, the cells were cultured in the presence or absence of cPrG·HCl (1 μM) or zLLLH (10 μM) for 6 h. NF-κB Activity was measured by the luciferase assay using dual assay kit. The results are presented as mean ± S.D. from three independent experiments.

presence of cPrG·HCl reflects the blockage of NF-κB activation.

We next examined the effect of cPrG·HCl on the nuclear translocation of NF-κB. Nuclear extracts were prepared from cells stimulated with TNFα in the presence or absence of cPrG·HCl, and were analyzed by Western blotting with antibodies against the NF-κB subunits, p65 and p50 (Fig. 2F). TNFα treatment increased the amount of both p65 and p50 in the nuclear extracts. zLLLH severely reduced the translocation of both subunits, as expected. In contrast, cPrG·HCl had no effect on the TNFα-induced increase of p65 and p50 in nuclear extracts, indicating that the nuclear translocation of NF-κB was not inhibited by cPrG·HCl. Consistent with these results, EMSA assays revealed that cPrG·HCl failed to inhibit the DNA binding of NF-κB in response to TNFα treatment (Fig. 2G). In addition, cPrG·HCl did not inhibit the DNA binding of NF-κB in vitro (data not shown).

PMA, is also known to activate NF-κB. As shown in Fig. 3, although cPrG·HCl did not suppress the nuclear translocation and the DNA binding of p65, cPrG·HCl again inhibited PMA-induced NF-κB activation in a dose-dependent manner similar to the inhibition of TNFα-induced NF-κB activation by cPrG·HCl. Thus, cPrG·HCl also inhibited specifically the PMA-induced transcriptional activation by NF-κB.

### *3.3. cPrG·HCl suppresses the NF-κB-dependent gene expression induced by Rac1, Cdc42, MEKK1, IKKα, IKKβ and p65*

It is of particular interest to determine if cPrG·HCl suppresses the transcriptional activity of NF-κB. To address this point, we transfected the cells with a reporter plasmid of NF-κB together with an expression plasmid encoding either the constitutively active mutants, IKKαEE and IKKβEE, or p65. As shown in Fig. 4A, NF-κB activity was enhanced more

than 5-fold in cells expressing either IKKαEE or IKKβEE, and more than 8-fold in cells over-expressing p65. zLLLH partially but significantly suppressed the IKKαEE- or IKKβEE-induced activation of NF-κB. Since zLLLH was added after the transfection followed by an incubation period required for the expression of IKKαEE and IKKβEE genes, NF-κB was already fully activated and hence the inhibition by zLLLH was apparently partial. cPrG·HCl reduced the IKK·EE- or IKKβEE-induced activation of NF-κB more potently than zLLLH. Furthermore, cPrG·HCl markedly suppressed the NF-κB-dependent gene expression in cells over-expressing p65. We further analyzed the effects of cPrG·HCl on NF-κB in the cells transfected with an expression plasmid encoding either the constitutively active mutants Rac1 and Cdc42, or MEKK1, which are also known to activate NF-κB [28–30]. cPrG·HCl again markedly suppressed the Rac1-, Cdc42-, and MEKK1-induced activation of NF-κB (Fig. 4B). Combining these results, it is obvious that cPrG·HCl suppresses NF-κB activation at the transcriptional level rather than at the upstream cellular signalling pathways.

## 4. Discussion

Here we demonstrated that cPrG·HCl alone caused relatively little cytotoxicity in HeLa cells. However, it enhanced TNFα-induced apoptosis, suggesting that cPrG·HCl progressively enhances the apoptotic process by suppressing the transcriptional activity of NF-κB. It should be noted that cPrG·HCl suppressed TNFα-induced NF-κB activation not only in HeLa cells but also in human astrocytoma U373 cells (data not shown). Furthermore, cPrG·HCl inhibited NF-κB activation induced by PMA not only in HeLa cells but also in U373 cells and COS7 cells (data not shown), indicating that the suppressive effect on NF-κB is ubiquitous. It has been reported that PNU156804, an analogue of prodigiosin 25-C, blocks IL-2-dependent proliferation by suppressing NF-κB activation in primary cultured human T-lymphocytes [24]. An apparent difference is that the inhibition of NF-κB by PNU156804 was due to the attenuation of IκBα and IκBβ degradation specifically in human lymphocytes, whereas cPrG·HCl inhibits neither the degradation of IκBα nor the concomitant nuclear translocation and DNA binding of NF-κB. Although the difference in the mechanism of the inhibitory action between cPrG·HCl and PNU156804 in these cell types is unclear, it is obvious that prodigiosin family compounds act as inhibitors of NF-κB activation in response to a wide range of extracellular stimuli in various cell types.

So far, several inhibitors of NF-κB activation have been reported. Among them, anti-inflammatory agents such as aspirin [31,32], sodium salicilate [33], and an indomethacin-related sulindac [34] are known to suppress NF-κB-dependent gene expression. Also, various natural products such as prostaglandin [35], curcumin [36], capcisin [37], and silymarin [38] are inhibitors of NF-κB activity. These agents preferentially reduce IκB degradation by inhibiting IKK activity and/or by attenuating the cellular signalling system linked to NF-κB activation. Furthermore, arsenite [39] and thiol metal compounds such as auranofin [40] directly inhibit IKK activity thereby suppressing NF-κB activation. In this context, the mode of action of cPrG·HCl is clearly different from that of these inhibitors since the TNFα-induced IκB degradation and IKK kinase activity are not affected by cPrG·HCl as shown in Fig. 2D,E. Rather, our study indicated that cPrG·HCl suppressed NF-κB-mediated gene expression without attenuating upstream signalling pathways.

In contrast, it has been revealed that several molecules suppress NF-κB-enhanced gene expression without inhibiting IKK activity, IκB phosphorylation, or the nuclear translocation of p65. Among them, a class of pyridinyl imidazoles, SB203580, suppresses NF-κB-dependent gene transcription by inhibiting p38 MAP kinase which phosphorylates TFIID (TATA-binding protein, TBP) [41]. This is also clearly different from the action of cPrG·HCl since cPrG·HCl did not inhibit p38 MAP kinase (data not shown). Conversely. both an immunosuppressant PG490 (Triptolide) [42] and chromium (VI) [43] inhibit the transcriptional activation by NF-κB in the absence of inhibition of nuclear translocation and DNA binding, indicating that these inhibitors act on the transcriptional machinery of NF-κB. The characteristics of inhibition by cPrG·HCl suggest that this compound also acts on the transcriptional step(s), resulting in the suppression of NF-κB-dependent gene expression.

Although it is still unclear how cPrG·HCl suppresses the transcriptional activation by NF-κB, our results allow the proposal of several plausible target(s) of cPrG·HCl. When NF-κB binds to the DNA target sequence, the p65 subunit recruits transcriptional coactivator molecules, such as the cAMP response element-binding protein (CBP) and p300, and general transcription factors such as RNA polymerase II [44,45]. Furthermore, it has been reported that LPS induces protein kinase A-dependent phosphorylation of p65 at serine 276 residue in T and B cell lines [46], of which phosphorylation leads to the recruitment of CBP and p300 [47]. As cPrG·HCl does not inhibit the transcription of the luciferase reporter gene under the control of the CMV promoter (Fig. 2B), the activities of general transcription factors are not suppressed by cPrG·HCl. Rather, it is more plausible that cPrG·HCl interferes with the specific interaction between p65 and these transcriptional coactivators or general transcription factors. Alternatively, cPrG·HCl possibly suppresses the activity of transcriptional factors associated with NF-κB.

Suppression of NF-κB activity severely disturbs the immune system, and also suppresses cell transformation, and we have observed that cPrG·HCl acts as an immunosuppressant and an anti-tumor drug. We conclude that the suppression of NF-κB by cPrG·HCl might confer the immunosuppressant and anti-tumor effects of this prodigiosin molecule.

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