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Invited review

The association of vitamins C and K_3 kills cancer cells mainly by autoschizis, a novel form of cell death. Basis for their potential use as coadjuvants in anticancer therapy

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Abstract

Deficiency of alkaline and acid DNase is a hallmark in all non-necrotic cancer cells in animals and humans. These enzymes are reactivated at early stages of cancer cell death by vitamin C (acid DNase) and vitamin K_3 (alkaline DNase). Moreover, the coadministration of these vitamins (in a ratio of 100:1, for C and K_3, respectively) produced selective cancer cell death. Detailed morphological studies indicated that cell death is produced mainly by autoschizis, a new type of cancer cell death. Several mechanisms are involved in such a cell death induced by CK_3, they included: formation of H_2O_2 during vitamins redox cycling, oxidative stress, DNA fragmentation, no caspase-3 activation, and cell membrane injury with progressive loss of organelle-free cytoplasm. Changes in the phosphorylation level of some critical proteins leading to inactivation of NF-κB appear as main intracellular signal transduction pathways. The increase knowledge in the mechanisms underlying cancer cells death by CK_3 may ameliorate the techniques of their in vivo administration. The aim is to prepare the introduction of the association of vitamins C and K_3 into human clinics as a new, non-toxic adjuvant cancer therapy.

Keywords: Autoschizis; Oxidative stress; Vitamins C and K_3; Cancer

1. Introduction

Cancer is characterised by cell cycle deregulation, progressive loss of cell differentiation and uncontrolled growth. It is the second leading cause of death in the world, for instance, 539 508 cases of deaths occurred in USA during 1996 (23.3% vs. 31.7% heart disease); and it is estimated to reach 555 500 in 2002. In Europe, the number of persons dying by cancer is estimated at 750 000. From a biochemical point of view, cancer cells have some remarked features: they are deficient in DNase activity [1], they have low activities of antioxidant enzymes [2], they show high rates of glycolysis [3], and most of cancer cells accumulate vitamin C [4].

An inhibition in the activity of both alkaline DNase (DNase I, EC 3.1.21.1) and acid DNase (DNase II, EC 3.1.22.1) has been reported in non-necrotic cancer cells at early stages of experimental carcinogenesis [5]. On the other hand, the reactivation of these enzymes has been observed in the early stages of spontaneous and/or induced tumour cell death [6]. Therefore, the use of compounds able to activate such endonucleases opens a novel therapeutic approach for cancer treatment. Since vitamins C and K_3 reactivate acid and alkaline DNases,

Abbreviations: TLT, transplantable liver tumour; NF-κB, nuclear factor kappa B; GSH, reduced glutathione; LDH, lactate deshydrogenase.

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respectively [7], the question was raised if the association of vitamins C and K_3 is of potential interest in cancer. Actually, it is known that vitamin C is cytotoxic against malignant melanoma cells, human leukaemia cells, neuroblastoma cells, tumour ascites cells, acute lymphoblastic leukaemia, and epidermoid carcinoma [8–12]. Furthermore, vitamin K_3 is cytotoxic against tumours of breast, stomach, lung, colon, nasopharyngeal, cervix, liver, leukaemia, and lymphoma cell lines [13,14]. It should be underlined that both vitamins are able to induce either apoptosis or necrosis depending upon the dose, the incubation time, and the cell type utilised [15,16]. So, combined vitamins C and K_3 at ratio of 100:1 after in vivo administration in tumour-bearing mice produced the following effects:

- Cancer growth inhibition in transplantable liver tumour (TLT)-bearing mice with an increase in life span (ILS) of 45.8%. Neither vitamin C nor vitamin K_3 administered alone has any significant effect on the life span of TLT-bearing animals [17].
- Selective potentiation of tumour chemotherapy. For instance, while cyclophosphamide alone, at a single sub-therapeutic dose of 80 mg kg^{-1} body weight increased the life span by 23%, its association with CK_3, increased the life span by 59.5% [18].
- Sensitisation of tumours resistant to some drugs. The pretreatment of TLT-bearing mice with CK_3 before injection of Oncovin increases the life span by 97.3% [19].
- Potentiation of the radiotherapy effects (20 Gy X-rays local irradiation) in mice bearing a solid form of intramuscularly transplanted TLT tumour [20].

Histopathological examinations of CK_3-treated mice did not indicate any sign of toxicity in normal organ and tissues, and some in vitro studies show that the addition of catalase (CAT) totally suppressed the effects of these vitamins association [19]. Therefore, we suggest that a redox cycling between both vitamins and the induced oxidative stress may explain the specific cytotoxic effects on cancer cells. In solution, vitamin K_3 is non-enzymatically reduced by vitamin C to form dehydroascorbate and the semiquinone-free radical. Such a semiquinone is rapidly reoxidised to its quinone form by molecular oxygen thus generating reactive oxygen species such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^\bullet$). Since CAT has a suppressive effect, H_2O_2 is likely to be the oxidising agent involved in the cytotoxicity by CK_3. Nevertheless, the precise mechanism which leads to cell death by CK_3 is still unknown and it has yet to be fully elucidated. Indeed, in addition to necrosis and apoptosis, several other types of cell death may exist, namely autoschizis, paraptosis and oncosis.

2. Cytotoxicity and oxidative stress by CK_3

We have recently shown that the association of vitamins C and K_3 induced a time- and dose-dependent cytotoxic effect [21]. Moreover, the cell death was only seen when both vitamins were added simultaneously, but any cytotoxic effect occurred when each vitamin was added alone. This synergistic effect clearly indicated that redox cycling is a major event in the mechanism of cytotoxicity induced by CK_3, followed by H_2O_2 generation and further oxidative stress. In addition to redox cycling, vitamin K_3, a naphtoquinone with a double bond α to a keto group, can undergo a Michael addition to form adducts with sulfhydryls and primary amines leading to cell injury and cell death. To discriminate which of both pathways (redox cycling or covalent binding) are involved in the cytotoxicity induced by CK_3, we used DMNQ (2,3-dimethoxy-naphtoquinone), a vitamin K_3 analog without arylation sites (see Fig. 1).

The association of vitamin C with DMNQ instead of vitamin K_3 produced the same profile of cytotoxicity as observed with CK_3, underlining the key role of the redox cycling pathway [21]. The use of other quinone moiety-bearing compounds, such as naphtoquinone and plumbagin, has clearly shown that reactive oxygen species are being generated indeed by redox cycling between vitamin C as reducing equivalent supplier and the quinone compound as catalyst. A strong relationship was observed between the half-redox potentials and the cytotoxic capacity of such compounds (data not shown). Moreover, some experiments to modulate the activity of CK_3 at different levels in the generation of reactive oxygen species were performed (Fig. 2). They included the addition of different antioxidants like CAT (to destroy H_2O_2), desferal (a transition metal chelator rendering ionic-free iron unavailable for a Fenton reaction), mannitol (to scavenge hydroxyl-free radicals), and *N*-acetylcysteine (NAC) (a precursor of GSH). These studies lead to the conclusion that H_2O_2 is likely the oxidising agent involved in the cytotoxicity induced by CK_3 [21].

If reactive oxygen species are being generated during CK_3 vitamins redox cycling, the cellular antioxidant status become a critical issue to explain CK_3 cytotoxicity. We have measured some parameters reflecting both energetic and redox status in three selected cell lines: a solid tumour TLT (a murine hepatoma cell line); and two non-solid tumours, Molt4 (acute lymphoid leukaemia cells, essentially neoplastic lymphocytes T) and K562 (chronic myeloid leukaemia cells, characterised by the Philadelphia chromosome). Among them, K562 cells have the highest level of GSH, ATP, and antioxidant enzyme activities. Most probably, due to all these signs, they were more resistant to the cytotoxic effect of CK_3 (Table 1).

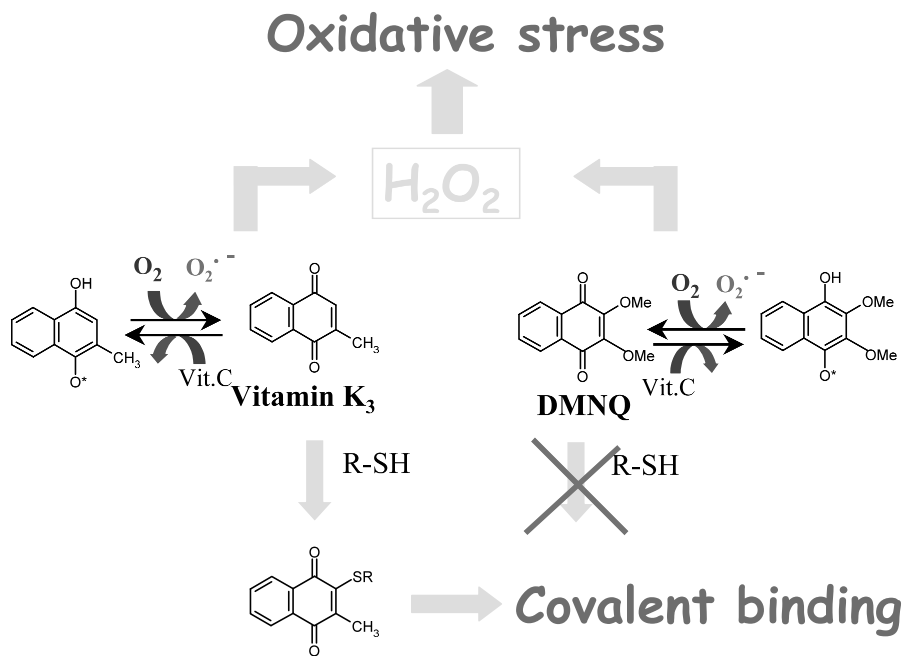

Fig. 1. Redox cycling or covalent binding? Vitamin K_3 can undergo futile oxidation–reduction cycling and thereby produce reactive oxygen species. Alternatively, it can form adducts with RSH leading to cell injury. By using DMNQ, a structural analog of K_3 without arylation sites, it is possible to distinguish if cytotoxicity induced by CK_3 is mediated by reactive oxygen species or by covalent binding.

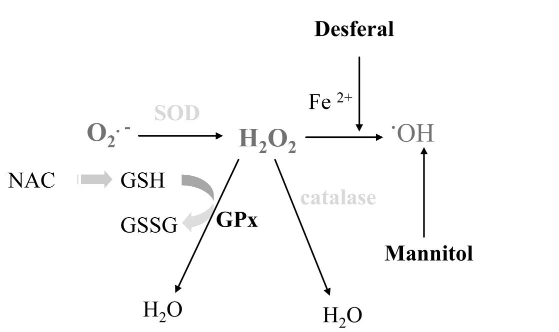

Fig. 2. Which reactive oxygen species is involved in CK_3 cytotoxicity? Modulation of the cytotoxic effect induced by these vitamins was performed by using enzymatic and non-enzymatic antioxidants. SOD dismutates $O_2^{-\bullet}$ in water and H_2O_2. CAT transforms H_2O_2 in water and oxygen. NAC is an antioxidant and serves as GSH precursor, by these means it helps in the transformation of H_2O_2 by GSH peroxidase. Desferal due to its metal chelating properties, rends iron unavailable for a Fenton reaction and blocks the formation of $HO^\bullet$. Mannitol, is a hydroxyl-free radical scavenger, avoiding the deleterious effects of this highly reactive oxidising agent.

Table 1
Energetic and antioxidant status of three different cell lines under basal conditions and sensibility against the association of CK_3 (2 mM/20 μM)

Markers	Cell lines		
	K562	Molt4	TLT
ATP (nmol (mg protein)$^{-1}$)	10.5±1.5	5.9±0.9 [a]	5.7±0.8 [a]
GSH (nmol (mg protein)$^{-1}$)	22.1±3.6	11.2±1.9 [a]	7.5±0.4 [a,b]
SOD (U (mg protein)$^{-1}$)	1.8±0.1	2.6±0.2	2.4±0.5
CAT (mU (mg protein)$^{-1}$)	41.5±2.1	34.2±1.2 [a]	3.0±0.5 [a,b]
GSHpx (mU (mg protein)$^{-1}$)	50.3±3.3	52.9±4.4	5.4±0.2 [a,b]
Cell survival (%)	80.0±5.2	37.5±4.5 [a]	9.6±2.7 [a,b]

Cells were incubated for 1 h in the absence of vitamins. Afterwards aliquots of cell suspension were taken and parameters were measured according to standard methodologies: ATP by using the bioluminescence kit from Boehringer; GSH by OPT method [40]; SOD by recording the reduction of NBT [41]; CAT by using $TiSO_4$ method [42]; and GSHpx by following NAPDH oxidation [43]. To assess cell survival, cells were incubated for 6 h in the presence of vitamins C and K_3 and survival was evaluated by measuring LDH leakage according to Wroblesky and Ladue [44]. The ratio between the activity within the cells and LDH leaked out was used as % of cell death. Survival was then 100 minus the % of cell death.
[a] $P < 0.05$ as compared with K562-treated cells.
[b] $P < 0.05$ as compared with Molt4-treated cells.

Thus, such a differential sensitivity to CK_3 seems to be associated to the cellular antioxidant and energetic status. For instance, by comparing our measurements of endogenous activities of superoxide dismutase (SOD), CAT and glutathione peroxidase (GSHpx) in TLT cells, with those activities reported in normal non-transformed cells [2], it results that enzyme activities in TLT cells represented about 5% of the non-transformed murine hepatocytes. Moreover, according to recent reports, the cytotoxicity induced by CK_3 exhibits a rather selective effect on cancer cells because human foreskin fibroblasts [22] and human gingival fibroblast [23] were highly resistant to CK_3 as compared with transformed cell lines.

3. How the association of CK_3 kills the cells?

Both morphological examination (Giemsa staining) and flow cytometry analysis (in cells loaded with Annexin-V and propidium iodide) allow to precise whether cells are dying by necrosis or apoptosis. On the basis of these parameters, it is suggested that cancer cells treated by CK_3 die principally by a particular form of cell death, sharing some characteristics of both necrosis and apoptosis [21]. In addition, preliminary results obtained in our laboratory suggest that DNA strand breaks induced by CK_3 (as shown by TUNEL procedure) did not correspond to DNA fragmentation as observed when apoptosis is occurred. Indeed, cell death whatever its type affects the genomic DNA integrity. During necrosis the induction of unspecific nucleases yields random sizes of DNA fragments that are visualised by a DNA smear. In apoptosis, however, 180–220 bp DNA laddering is the result of an active and specific endonuclease, the caspase-activated DNA fragmentation factor caspase-3-activated DNase, the so-called DFF40/CAD. In different cell lines (such as TLT, K562, Molt4), the activation of caspase-3 (a hallmark of apoptosis) did not occur in CK_3-treated cells. The caspase family members exist as procaspases that are activated after an aspartate residue cleavage. Therefore, we have hypothesised that CK_3 combination may induce the proteolysis of the procaspase-3, but the oxidation by H_2O_2 of a critical cysteine residue in QACRG motif of the caspase catalytic site renders the enzyme inactive. Altogether, these observations supported the conclusion that cancer cells treated by CK_3 are dying mainly by **autoschizis**, a new type of cell death previously described by Gilloteaux and coworkers [24–27]. For instance, in experiments with human T24 prostate cells treated for 1 h with CK_3 (2 mM/20 μM), cell population has been depleted by 25%, and this decrease in cell population is accompanied by changes in cell morphology: cells appear as if they are dividing (Fig. 3). The most remarkable events that can be detected are: (1) a delocalisation of organelles around the nucleus thus leaving a cytoplasm empty of them, (2) a process of self-excision of organelle-free cytoplasm, and (3) a diminution of the overall size of the tumour cells. Interestingly, this type of cell death may be more frequent as initially has been thought. Indeed, it is not a cell type-specific phenomena since it has been observed in a wide variety of cells including murine hepatomas (TLT), human leukemias (Molt4 and K562), and human urologic cell lines (T24 and DU145). Moreover, it seems to be not specific for the association of CK_3 since another form of cell death, the so-called Blister cell death/oncosis [28], show remarked morphological features close to autoschizis and lack of caspase-3 activation as well. These authors used sanguinarine, an antitumour and anti-inflammatory drug against a wide variety of human cell lines including K562 cells. In the absence of a morphological characterisation other than that performed [24–27], we do not have still enough information to conclude whether other forms of cell death, such as paraptosis [29] or aponecrosis [30], look like autoschizis. This morphological analysis is absolutely required since several cell death processes are already occurring in the absence of caspase-3 activation [31–34], but they are still considered by several authors as apoptosis. For instance, the apoptosis inducing factor (AIF) has been reported to be a redundant pathway leading to apoptosis without activation of caspases [35].

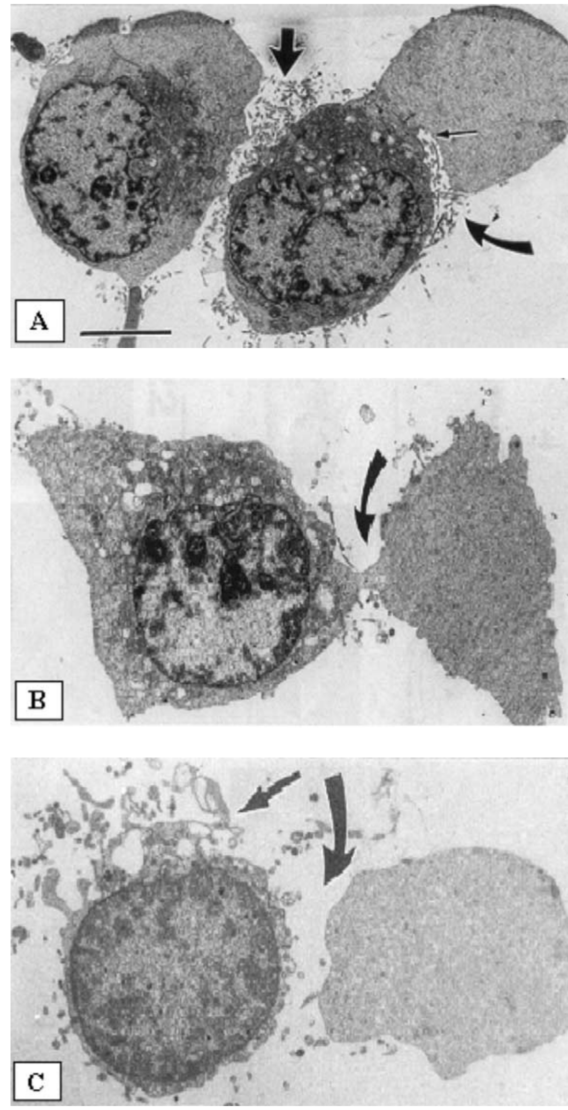

Fig. 3. T24 cells death by autoschizis after 1 h treatment with CK_3. Transmission electron microscopy (TEM) of cell doublet structures. The excising regions are free from organelles (A), a narrow cytoplasmic bridge still links the excising cytoplasm to the remaining cell body (B), and finally a perikaryon formed by cytoplasmic self-excision (C). Notice the narrow rim of remaining cytoplasm. Scale = 5 μm. (This figure is a kind gift of Dr. J. Gilloteaux).

Autoschizis is then a novel type of cell death which is caspase-3-independent and it is characterised by cell membrane damage with progressive excision of organelle-free cytoplasm. It may be raised, however, that such a cell death is rather an incomplete apoptotic program, due for instance to the oxidation of cysteinyl residue in caspase catalytic site that renders the enzyme inactive. Another possibility is that depletion of ATP is too much extensive avoiding the formation of the apoptosome complex. Nevertheless, the morphological analysis as well as the profile observed with FACScan of Annexin-V loaded cells, lend to support the idea that autoschizis is the predominant form of cell death induced by CK_3 (and perhaps by other compounds) and may complement apoptosis in antitumour surveillance.

4. Which signal transduction pathways is involved in autoschizis?

It is generally accepted that protein kinase cascades play a major role in controlling cell function and differentiation, including cell death. In that sense, we have previously reported that CK_3 induce a G1 block in the cell cycle [25]. Furthermore, sodium orthovanadate, a well-known inhibitor of protein tyrosine phosphatases, completely suppresses the cytotoxicity induced by CK_3 [21]. Sodium orthovanadate may reduce CK_3 cytotoxicity by acting in three different ways: the first one involves a redox reaction between vanadate and vitamins. This possibility is very unlikely due to the involved redox potentials. Moreover, vanadate did not interfere with oxygen uptake of vitamin mixture as measured with a Clark electrode (manuscript in preparation). The second possibility is related to the possible reaction of orthovanadate with H_2O_2 to form peroxovanadate. Such a reaction may deplete the cell of H_2O_2 leading to a decrease of CK_3 cytotoxicity that could explain the protective effect of vanadate. The third possibility is that vanadate by inhibiting tyrosine phosphatases, modifies the phosphorylation state of some critical protein (Fig. 4).

Which transcription factors are involved in the intracellular signals triggered by CK_3? Among different transcription factors, NF-κB was selected because it is a

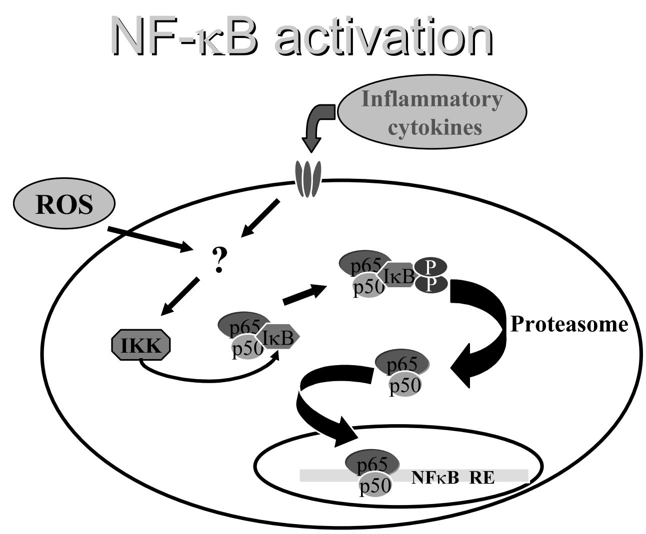

Fig. 5. Activation of NF-κB. Homo- or heterodimers of NF-κB are inactive under resting conditions by binding to IkB. After appropriate stimulus (reactive oxygen species or inflammatory cytokines), a protein kinase is activated (IKK) which phosphorylates IkB. Once the inhibitor is phosphorylated it is recognised by the ubiquitin system and further degraded by the proteasome thus releasing free in cytosol NF-κB which is then translocated into the nucleus, binds to DNA and activates the genes that it regulates.

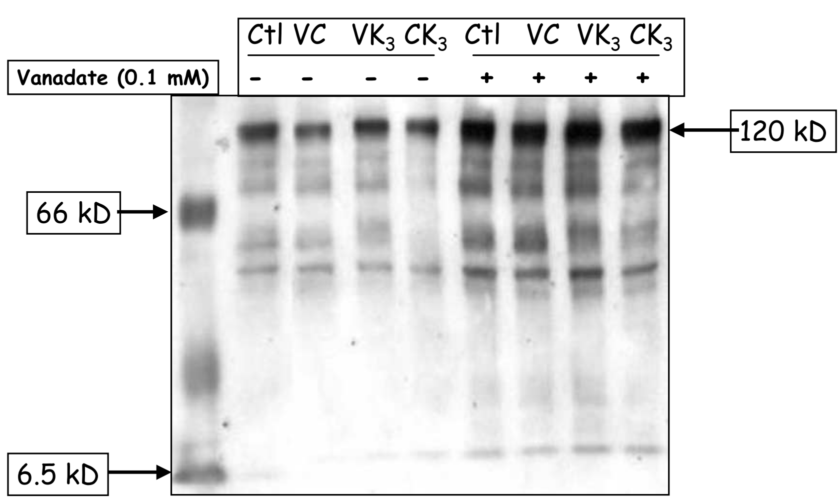

Fig. 4. Effect of vanadate in the phosphorylation levels of tyrosine residues in CK_3-treated cells. Cells were incubated for 1 h with vitamin C (2 mM) and vitamin K_3 (10 μM) either alone or in combination, and in the absence or in the presence of sodium vanadate (0.1 mM). Afterwards cells were lysed and proteins of the supernatant obtained after centrifugation were submitted to electrophoresis (8% acrylamide). Proteins were transferred to nitrocellulose membranes and probed against anti-phosphotyrosine antibodies. Bands were visualised by using ECL and further film revelation.

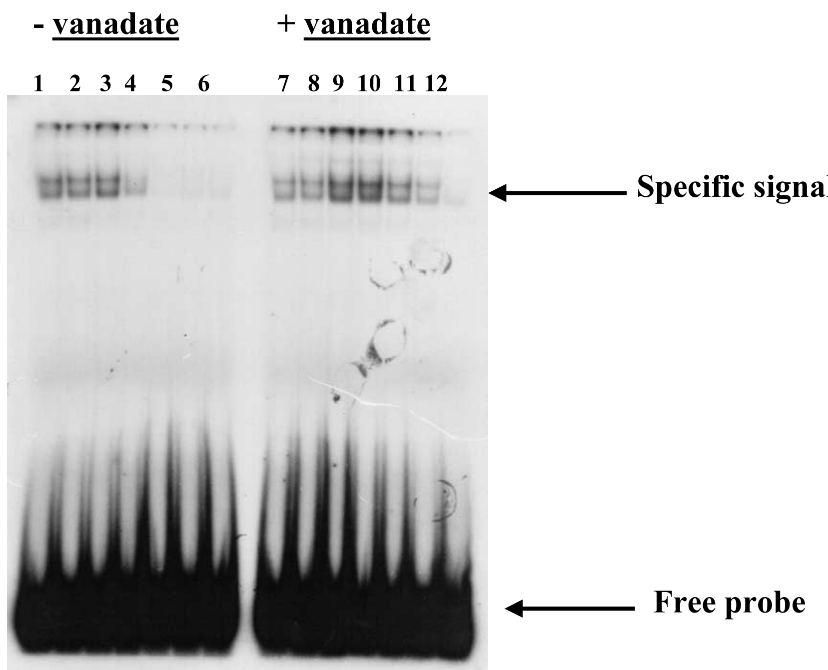

Fig. 6. NF-κB DNA binding activity. Cells were incubated for 2 h (lanes 1, 4, 7, 10), 4 h (lanes 2, 5, 8, 11), and 6 h (lanes 3, 6, 9, 12) with a combination of vitamins C (2 mM) and vitamin K_3 (10 μM), in the absence (lanes 1–6) or in the presence (lanes 7–12) of sodium vanadate (0.1 mM). The nuclear extracts were submitted to electrophoretic mobility shift assay (EMSA) using a double-stranded ^{32}P-labeled 45-mer NF-κB oligonucleotide from HIV terminal repeat, 5′-GGTTACAAGGGACTTTCCGCTG-3′. The gel was dried and visualisation of radioactive bands was carried out by film exposition.

pleiotropic transcription factor involved in cell death and proliferation. Since H_2O_2 (a major mediator in cancer cell death by CK_3) induces the activation of NF-κB, and due to the protective effects shown by both vanadate (protein phosphorylation?) and the antioxidant NAC, as previously reported [21], the question about the involvement of NF-κB in CK_3 cytotoxicity has been raised. It has been proposed that NF-κB inhibits apoptosis and favours cancer cell survival [36,37]. Fig. 5 shows that under physiological conditions in normal cells, NF-κB is present in its inactive state in the cytoplasm as a complex with its inhibitor I-κB. This latter is degraded by the proteasome after phosphorylation and ubiquitination reactions. This allows the release of NF-κB and its translocation to the nucleus, where it binds to the promoter region of DNA and activates genes that mediate carcinogenesis and metastasis [38]. The results obtained, however, show that CK_3 is rather inhibiting NF-κB than causing its activation (Fig. 6). This is not completely unexpected since cancer cells survival is associated with activation of NF-κB and other compounds inducing similar forms of cell death, like sanguinarine, are also able to inhibit NF-κB [39].

As conclusion, on the basis of the previous work as well as on the experiments currently under study, we expect to have some indications about the intracellular targets of CK_3 as well as concerning the signal transduction pathways involved in this particular cell death (autoschizis) induced by these vitamins. Every effort dealing with a concomitant increase in the therapeutic effect of anticancer drugs while reducing undesirable secondary effects is of a major importance in clinical practice today. A better knowledge on the mechanism through which CK_3 kills cancer cells may argue for an administration of vitamins C and K_3 **as adjuvants** in the classical protocols applied to **patients suffering from cancer**. Such adjuvant therapy will not produce **any supplementary risk** for the patients but, on the contrary it will lead to beneficial effects of clinical cancer treatment.

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