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In vitro Assessment of Renal Toxicity and Inflammatory Events of Two Protein Phosphatase Inhibitors Cantharidin and Nor-Cantharidin*

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Abstract: In China, cantharidin has been reported to be active against various human cancers, but with severe side effects such as nephrotoxicity. In order to reduce this toxicity, its demethylated analogue nor-cantharidin has been synthesized and used in cancer therapy, but with only few data regarding safety assessment. The aim of this study was to compare the *in vitro* effects of cantharidin and nor-cantharidin on renal toxicity and on inflammatory events associated with tumoural process where protein phosphatases could be involved (energy status, prostanoid production, glutathione and nitrite contents) on RAW 264.7 and LLC-PK_1 cells. In macrophages, both cantharidin and nor-cantharidin decreased cell viability, in a concentration- and time-dependent manner. However, IC_{50} was lower with cantharidin than with nor-cantharidin. These two drugs significantly decreased the ATP level after 24 hr incubation. However, ATP decreased much more with cantharidin (up to 4 times) than with nor-cantharidin. When control macrophages were activated with lipopolysaccharide+interferon-γ for 24 hr a significant increase in nitrite content and in prostanoids were observed. Addition of either drug decreased nitrite generation and prostanoids, however these decreases were greater with cantharidin than with nor-cantharidin. In LLC-PK_1 cells, incubated with either cantharidin or nor-cantharidin, our results show significant differences between the two drugs, similar to those observed in peritoneal macrophages, except for GSH content with opposite variations in both cells. We provide a better understanding of the various mechanisms of cantharidin side effects, allowing an easier comparison with nor-cantharidin which could be an attractive therapeutic potential in cancer chemotherapy in western countries.

The active constituent of *Mylabris*, cantharidin, is produced by different species of blister beetles (Oaks *et al.* 1960). Cantharidin and its analogues have diverse actions. They are antitumour agents, pesticides and purported aphrodisiacs and continue to result in human poisoning today. Moreover, severe nephrotoxicity has been observed (Wang 1989; Polettini *et al.* 1992). Renal dysfunction is common and related to acute tubular necrosis and glomerular destruction (Karras *et al.* 1996). Despite many studies on the physiological and biochemical effects of cantharidin, there is limited information on its mechanism of action. Besides interference with mitochondrial respiration, the toxic effects of cantharidin and its analogues are attributable to their affinity and specificity for a cantharidin-binding protein identified as protein phosphatases (Bagatell & Dimitrov 1965; Li & Casida 1992; Honkanen 1993). Cantharidin and structural derivatives have antitumour activity against various cancer cell lines, ascites hepatoma in mice and many of them showed selective cytotoxicity for colon tumour cell lines (Walter 1989; Laidley *et al.* 1997; McCluskey *et al.* 2002; Peng *et al.* 2002). Protein phosphatase is present in many mammalian tissues and is involved in regulatory phosphorylation-dephosphorylation events which modulate multiple cellular functions, ion channels and enzymatic activities (Taylor *et al.* 1999 & 2000). Inhibition of protein phosphatase activity may account for the diverse effects and toxicity of these compounds. Although cantharidin induces tissue-specific changes in the phosphorylation state of several phosphoproteins, it is difficult to establish the initiating event in protein phosphatase inhibitory toxicity (Eriksson *et al*. 1992; Eldridge & Casida 1995).

Nor-cantharidin, a synthetic demethylated analogue of cantharidin, is now in use in China as a routine anticancer drug against different kinds of digestive tract cancers, such as hepatoma, oesophagus carcinoma and gastric cancer (Wang 1989; Liu *et al.* 1995; and personal communication) rather than cantharidin, because the two methyl groups of cantharidin are not the main functional groups for antitumour activity, and with a view to reducing nephrotoxic side effects. Nor-cantharidin also stimulates the bone marrow production of white cells, but without kidney toxicity. However, few data on risk assessment and on the exact cellular and molecular action mechanisms of nor-cantharidin on tu-

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mour cells have been published to date (Sun *et al.* 2000; Chen *et al.* 2002).

Macrophages have been dually implicated in tumour development. On the one hand, these cells are capable of phagocytosis towards tumour cells and of secretion of tumour-growth inhibitory cytokines. On the other hand, activated cells can induce transformation of normal cells *in vitro* and a close association has been established between chronic inflammation and tumour incidence (Fulton & Chong 1992; Fitzpatrick 2001; Shacter & Weitzman 2002; Jakobisiak *et al.* 2003). The increased release of reactive oxygen species or nitric oxide can be responsible for the DNA damage and neoplastic transformation. In addition, significant evidence has been accumulated suggesting that the inducible form of cyclooxygenase (COX-2), a central enzyme in the prostaglandin biosynthesis pathway, which is induced by proinflammatory and mitogenic stimuli (Diaz-Guerra *et al.* 1999) plays a complex role in tumour development (Tsujii *et al.* 1997 & 1998; Bol *et al.* 2002; Gasparini *et al.* 2003). Activation of macrophages to produce a variety of biologically active molecules targeting tumour cells has been shown to require various protein kinases and phosphatases (Novotney *et al.* 1991; Pixley *et al.* 2001; Comalada *et al.* 2003). The inhibition of protein phosphatase activities may play several crucial regulatory roles and the ability to inhibit the activity of these protein phosphatases underlies the toxicity of cantharidin and of its structural analogues on cancer cells but probably also on normal kidney cells.

The aim of this study was to compare the *in vitro* effects of cantharidin and nor-cantharidin on renal toxicity and on inflammatory events where protein phosphatases could be involved (energy status, prostanoid production, glutathione and nitrite contents). For this purpose we used the porcine renal tubular cell line LLC-PK_1 with proximal tubular properties and mouse peritoneal macrophages cell line (RAW 264.7) activated by lipopolysaccharide + interferon-γ. Raw 264.7 cell line was used as a macrophage model, because these cells share phenotypic and functional features with normal macrophages (Raschke *et al.* 1978; Blonska *et al.* 2003).

Materials and Methods

Materials. Cantharidin, lipopolysaccharide and MTT were purchased from Sigma (France). Nor-cantharidin was synthesized by G.S. Wang (Wang 1989). Foetal calf serum, recombinant interferon (IFN-γ), NADPH, GSH, oxidised GSH and glutathione reductase were obtained from Boehringer Mannheim (France). DMEM, RPMI and additional medium supplements were ordered from Life Technologies. 2-vinyl-pyridine was obtained from Merck (France). All other chemicals were of the highest grade of purity and commercially available. Stock solutions of cantharidin and nor-cantharidin were prepared in DMSO and stored at −20°. Before use these solutions were adjusted to final 0.1% DMSO in the medium.

Cell isolation, culture and treatment. LLC-PK_1 cells and macrophages RAW 264.7 (ATCC) were grown respectively in Dulbecco's MEM-glutamax (DMEM) or in RPMI medium supplemented with 10% foetal calf serum, 1% penicillin/streptomycin in a 5% CO_2–95% CO_2 atmosphere at 37°. Cells were seeded in 96-multiwell dishes at a density of 100,000 cells/ml (100 µl in each well) for viability studies, or in 6-well dishes (2 ml in each well) for measuring prostanoid, ATP, glutathione contents and the release of alkaline phosphatase, γ-glutamyltransferase, N-acetylglucosaminidase and lactate deshydrogenase. Except for cell viability study and ATP content, RAW 264.7 macrophages were exposed to the combination of lipopolysaccharide (50 ng/ml) and interferon-γ (100 U/ml), so-called MIX, for 24 hr. Macrophages and LLC-PK_1 cells were incubated either with cantharidin or with nor-cantharicin for 24 hr or 48 hr (1 to 100 or 600 µg/ml, respectively, for cell viability, 1 to 100 µg/ml for biochemical parameters). The comparison of effects was performed at equipotential concentrations and at concentration levels of relevance for anticancer potential as well as the duration of the exposure (Yang *et al.* 1997; Sun *et al.* 2000; McCluskey *et al.* 2003).

Cell viability assessement. Macrophages and LLC-PK_1 cells were incubated with cantharidin or nor-cantharicin in RPMI or DMEM without foetal calf serum and antibiotics for 3 hr to 48 hr. Cell viability was assessed by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue; MTT). The plates were read at 535 nm on a Multiskan plus spectrophotometer (Flow Laboratories). Results for the dose-response cytotoxicity assays are expressed as a percentage of the controls and IC_{50} values were calculated.

The release of the marker enzyme for apical membranes: alkaline phosphatase, γ-glutamyltransferase, for cytosol: lactate deshydrogenase, or lysosome: N-acetylglucosaminidase was determined in the culture medium (analytical kits, Boehringer, France). Enzyme activities are expressed as milliunits per 10^6 cells.

Nitric oxide production. Nitrite (NO_2^-) content in the culture supernatant was determined spectrophotometrically using a procedure based on the Griess reaction (Green *et al.* 1982). Sample aliquots were mixed with the Griess reagent and the absorbance at 550 nm was determined using a microplate reader (Multiskan plus, Flow Laboratories). NO_2^- concentrations were determined using sodium nitrite as standard. All data were normalized per 10^6 cells.

Prostanoid production. Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) (assayed through their respective hydrolysis product 6-keto-$PGF_1\alpha$ and TXB_2) released into the culture medium were assessed using ELISA kit (Neogen kits, Euromedex) according to manufacturer' s instructions.

ATP determination. RAW 264.7 were incubated 24 hr in RPMI without phenol red and treated with cantharidin and nor-cantharidin for 24 hr. Incubation was stopped by adding 0.6 M perchloric acid for deproteinization. After scraping the cells, ATP content was determined by high pressure liquid chromatography as previously described (Massicot *et al.* 1997). ATP is expressed as nmol/ml.

Table 1.

Effects of cantharidin and norcantharidin on cell viability determined by MTT assay.

	IC_{50} µg/ml			
	3 hr	6 hr	24 hr	48 hr
Macrophages RAW 264.7				
Cantharidin	20	7	0.79	0.90
Nor-cantharidin	>600	350	5.76	3.94
LLC-PK_1				
Cantharidin	>>100	>>100	7.43	3.18
Nor-cantharidin	>>600	>>600	75	43

Results are expressed as a time course of IC_{50} values. All experiments were repeated at least three times with similar results.

Table 2.

Effects of cantharidin and nor-cantharidin on TXB_2, PGI_2 and NO production in RAW 264.7 activated by MIX (LPS 50 ng/ml+IFNγ 100 U/ml).

	µg/ml	TXB_2 Pg/10^6 cells	$^{§}PGI_2$ Pg/10^6 cells	NO mmol/10^6 cells
Control		0.301±0.003	0.020±0.001	22.07±4.73
MIX		0.900±0.002$^{\$}$	0.410±0.006$^{\$}$	54.59±8.50$^{\$}$
MIX+Cantharidin	100	0.102±0.001*	0.038±0.006	25.35±4.69
	10	0.084±0.002*	0.024±0.002*	27.45±0.96
	1	0.190±0.020*	0.050±0.003	38.78±5.53
MIX+Nor-cantharidin	100	0.210±0.003*	0.110±0.008*	35.85±3.30*
	10	0.240±0.002*	0.112±0.016*	44.55±3.77*
	1	0.360±0.006*	0.330±0.011*	41.46±7.73*

Results are expressed as mean±S.D. values obtained from 6 assays per group, each performed in duplicate. Significance of differences: MIXversus control $^{\$}P<0.05$; cantharidin or nor-cantharidin versus MIX *P<0.05. §Measured through its hydrolysis product 6-keto-$PGF_1\alpha$. MIX: lipopolysaccharide 50 ng/ml+interferon γ 100 U/ml; TXB_2: thromboxane B_2; PGI_2: prostacyclin; no: nitric oxide.

Glutathione and related enzyme determination. After medium change with PBS containing 0.1% Triton X-100, the cells were scraped and stored at −80° until analysis. Reduced GSH and oxidized GSH were determined in cellular homogenates as described by Griffith (1980). GSH peroxidase and GSH reductase activities were measured according to Carlberg & Mannervik (1985) and Flohe & Gunzler (1984), respectively. Glutathione levels are expressed as nmol/10^6 cells and glutathione-related enzyme activities as nmol/mg protein. Protein content was determined according to Bradford (1976).

Data analysis. All results are presented as mean±S.D. (n=6). Analysis of prostanoid production, glutathione and nitrite contents were performed using the non-parametric Mann-Whitney test. Statistical significance was defined as $P<0.05$.

Results

In macrophages, both cantharidin and nor-cantharidin decreased cell viability, in a concentration- and time-dependent manner (table 1). However, cytotoxicity was more pronounced with cantharidin (7 times and 4 times after 24 hr and 48 hr incubation) than with nor-cantharidin. These two drugs significantly decreased ATP level after 24 hr incubation (results not shown). However, ATP decreased much more with cantharidin (up to 4 times) than with nor-cantharidin (0.32±0.01 nmol/ml versus 1.25±0.05 nmol/ml) at the higher dosage.

Treatment with MIX for 24 hr caused a significant increase in PGI_2 (20 times) and in TXB_2 (3 times) production, indicating the activation of inducible cyclooxygenase-2 (COX-2) enzyme (table 2). The production of PGI_2 and TXB_2 by MIX-stimulated macrophages was decreased by both cantharidin and nor-cantharidin. Treatment with cantharidin does not result in a higher increase of TXB_2 or PGI_2 synthesis than with nor-cantharidin (up to 73% and up to 77%, respectively) as compared to MIX.

In the absence of drugs, the basal nitrite production of the RAW 264.7 culture was 22.07±4.73 µM/10^6 cells. In response to 24 hr exposure to MIX, nitrite level increased to 54.59±8.50 µM/10^6 cells (147%) indicating the activation of inducible nitric oxide synthase enzyme (table 2). Both cantharidin and nor-cantharidin treatments resulted in a dose-dependent inhibition in MIX-induced nitrite generation (29% to 54% and 24% to 34%).

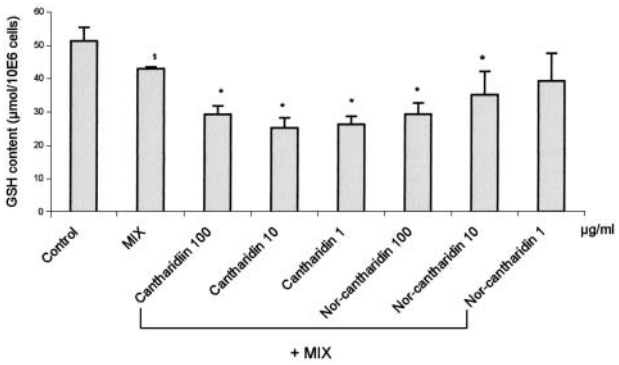

Fig. 1. Effects of cantharidin and nor-cantharidin on reduced glutathione in RAW 264.7. Results are expressed as mean±S.D., 6 assays per group. Significance of differences: MIX versus control, $^{\$}P<0.05$, cantharidin or nor-cantharidin versus MIX, *P<0.05. MIX: lipopolysaccharide 50 ng/ml+interferon 100 U/ml.

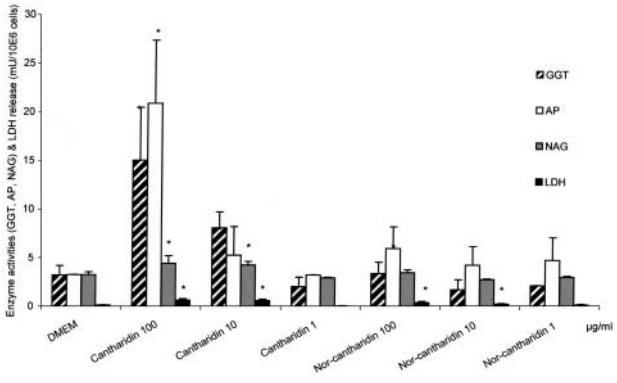

Fig. 2. Twenty-four hr effects of cantharidin and nor-cantharidin on GGT, AP, NAG and LDH release in LLC-PK_1 cells. Results are expressed as mean±S.D., 6 assays per group, each performed in duplicate. Significance of differences: cantharidin or nor-cantharidin versus control, *P<0.005. GGT: γ-glutamyltransferase; AP: alkaline phosphatase; NAG: N-acetylglucosaminidase; LDH: lactate deshydrogenase.

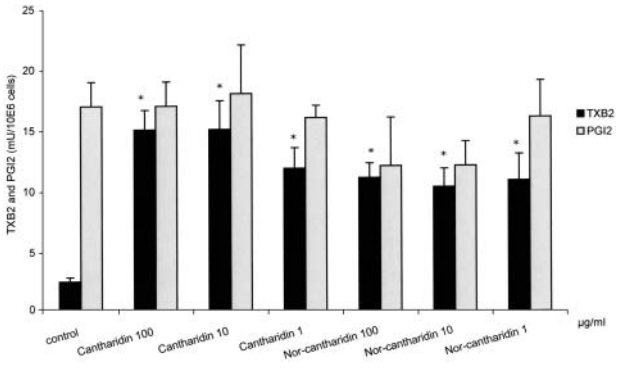

Fig. 3. Twenty-four hr effects of cantharidin and nor-cantharidin on thromboxane (TXB_2) and prostacyclin (PGI_2) production in LLC-PK_1 cells. Results are expressed as mean±S.D., 6 assays per group, each performed in duplicate. Significance of differences: cantharidin or nor-cantharidin versus control, *$P<0.005$. TXB_2: thromboxane B_2; PGI_2: prostacyclin.

GSH level was significantly decreased following MIX treatment (16%) and this decrease was further enhanced with nor-cantharidin (9% to 31%) and even more with cantharidin (32% to 39%) (fig. 1).

In renal LLC-PK_1 (as in macrophages), cantharidin reduced cell viability in a time- and concentration-dependent manner with a 3 hr IC_{50} very much higher than 100 µg/ml, decreasing progressively to 3.18 µg/ml by 48 hr (table 1). Further assays were standardized at 24 hr for convenience. Cytotoxicity was 10 to 13 times lower with nor-cantharidin than with cantharidin after 24 hr and 48 hr respectively. LLC-PK_1 cells were 10 times less sensitive than macrophages to the cytotoxic effect of both cantharidin and nor-cantharidin. Furthermore, the activities of γ-glutamyltransferase, alkaline phosphatase, N-acetylglucosaminidase and lactate dehydrogenase were significantly increased by cantharidin (10 and 100 µg/ml) (fig. 2). In contrast, in nor-cantharidin-treated cells enzyme release was similar to control cells except for lactate dehydrogenase activity which increased at two highest concentrations (10 and 100 µg/ml) and alkaline phosphatase activity doubled at 100 µg/ml.

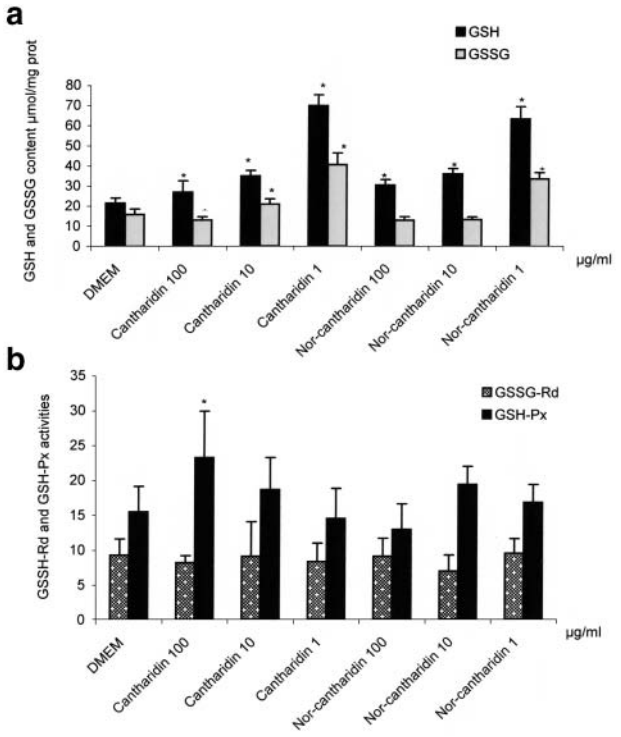

Fig. 4. Effects of cantharidin and nor-cantharidin on reduced (GSH) and oxidized (GSSG) glutathione (a) and on glutathione-related enzymes (b) in LLC-PK_1 cells. Results are expressed as mean±S.D., 6 assays per group, each performed in duplicate. Significance of differences: cantharidin or nor-cantharidin versus control, *$P<0.05$. GSSG: oxidised GSH; GSSG-Rd: glutathione reductase; GSH-Px: gluthathione peroxidase.

Cantharidin and nor-cantharidin did not produce a significant alteration in the release of prostacyclin ($PGFI_2$) at any concentration (fig. 3). In contrast, the release of TXB_2 was significantly higher with cantharidin (4.5 up to 6 times) than with nor-cantharidin (4 times whatever the concentration).

In LLC-PK_1 cells, cantharidin caused a significant concentration-dependent increase (up to 2.6 times) in oxidized and in reduced (up to 3 times) glutathione levels associated with an increase in glutathione peroxidase activity at the highest concentration (fig. 4). As in cantharidin-treated cells, GSH level was concentration-dependently increased in nor-cantharidin-treated cells. In contrast, oxidised GSH level remained similar to that measured in the control group, except with the highest concentration. There was no significant differences in glutathione reductase activity either with cantharidin or nor-cantharidin.

Discussion

The aim of this study was to analyse the potential cellular toxicity of cantharidin and nor-cantharidin, its demethylated analogue. We show that the cytotoxic potency of nor-cantharidin is 10 times less than that of cantharidin when incubated with RAW 264.7 macrophages and in LLC-PK1 cell lines. These results are consistent with previous observations obtained in various cancer cells (McCluskey *et al.* 2000; Sakoff *et al.* 2002). Decrease in cell viability and ATP depletion induced by cantharidin in peritoneal macrophages were observed at a much lesser degree with nor-cantharidin. By preserving energy status, nor-cantharidin leads to lesser metabolic disturbances than cantharidin, demonstrating a lower potential cytotoxicity. Although cantharidin and nor-cantharidin are equipotent inhibitors of protein phosphatase, with an approximate 10 times larger selectivity against protein phosphatase 2A (Honkanen 1993; McCluskey *et al.* 2002 & 2003; Sakoff *et al.* 2002), substantial differences were observed in regard to their cytotoxicity and it was suggested that other factors such as differences in cellular uptake or other intracellular targets may be involved.

Because an increased risk of cancer has been associated with inflammation and with up-regulation of the inducible

species of both iNOS and COX-2 (Vane 1998; Ambs *et al.* 1999; Beck *et al.* 1999), we have hypothesized that these two enzymes could be intracellular targets for cantharidin and nor-cantharidin. We confirm that activation of macrophages with MIX resulted in the release of both reactive nitrogen species such as nitric oxide and eicosanoids such as PGI_2 and thromboxane A_2 (Laskin & Pendino 1995) which could be a consequence of iNOS and COX-2 expression, respectively. The coordinated induction of both enzymes might be a cellular effect produced by a transcriptional activation, nuclear factor-κB (NF-κB), that controls the expression of these inducible enzymes particularly when MIX is the unique stimulus (D'Acquisto *et al.* 1997; Pahan *et al.* 1998; von Knethen *et al.* 1999). We have shown that the inhibition of PGI_2 and TXB_2 synthesis induced by inflammatory cytokines (MIX) is much greater with cantharidin than with nor-cantharidin. The inducible cyclooxygenase isoform (COX-2) in macrophages could be a differential molecular target for cantharidin and nor-cantharidin providing a possible mechanism for the interference of the drugs with the balance of vasoactive prostanoids. The greater inhibition found with cantharidin compared with nor-cantharidin could account for the greater toxicity of cantharidin. Whatever the mechanism involved, it can be concluded that the reported antitumoural activity of these protein phosphatase inhibitors could proceed from the inhibition of COX-2, as reported by others (Bol *et al.* 2002; Gasparini *et al.* 2003). Moreover, as it was previously shown, these results suggest that protein phosphatases play an important role in COX-2 expression and elevated prostaglandin levels (Park *et al.* 2001). The decrease we observed in eicosanoid production after cantharidin treatment can also be linked to reduced NF-κB activation.

In addition, our results on nitric oxide production confirm and demonstrate previous data, that the production of nitric oxide in stimulated RAW 264.7 cells can be suppressed by cantharidin, a specific inhibitor of protein phosphatase (Dong *et al.* 1995; Pahan *et al.* 1998). The ability of cantharidin to inhibit nitric oxide synthesis suggests that cantharidin may be an important regulator of the nitric oxide signalling pathway and nitric oxide-mediated cytotoxic processes. In contrast, nor-cantharidin which produces a lower nitric oxide depletion than cantharidin demonstrates a lower vasoconstrictor potential and a lower cytotoxicity.

Our results in macrophages show that cantharidin and nor-cantharidin decrease GSH level but the much lower GSH depletion with nor-cantharidin is indicative of a reduced free radical injury with this drug. However, the lack of concentration-response effect of cantharidin could be due to the fact that all three concentrations were above maximum. Various tumours are frequently infiltrated by large numbers of macrophages which contribute to carcinoma cell oxidative stress by producing oxygen radicals (Fulton & Chong 1992; Kundu *et al.* 1995; Sarti *et al.* 2002). Because protein phosphatases are also involved in myeloma cell growth by generating reactive oxygen species (Brown & Bicknell 2001; Kang & Choi 2001) nor-cantharidin, a protein phosphatase inhibitor which preserves energy status and prevents a strong GSH depletion, could exert its antitumoural activity by reducing hydroperoxide production and with a lower cytotoxicity comparatively with cantharidin.

It has been shown that glutathione depletion caused marked inhibition of cytokine-induced iNOS expression and activity in different cell types, indicating similar mechanisms of cellular redox regulation of cytokine-induced nitric oxide synthesis (Duval *et al.* 1995; Hothersall *et al.* 1997; Nikulina *et al.* 2000). Thus, the reduced nitric oxide and GSH levels we measured in our study lead us to conclude that GSH depletion could account for the inhibition of MIX-stimulated nitric oxide production in nor-cantharidin- and even more in cantharidin-treated cells.

The treatment of LLC-PK_1 cells with cantharidin for 24 hr produces cytotoxicity as demonstrated by the increase in GGT, alkaline phosphatase and N-acetylgluocosaminidase activities with an associated loss in cell viability. These observed *in vitro* variations are consistent with the *in vivo* observations reported by Karras *et al.* (1996) that cantharidine is related to acute tubular necrosis. In contrast, nor-cantharidin has no effect on enzyme release and although cell viability is decreased, this decrease remains significantly higher than with cantharidin-treated cells. This indicates that nor-cantharidin strongly attenuates membrane injury and may explain that nor-cantharidin is less cytotoxic than cantharidin. Moreover, treatment with cantharidin results in a higher increase of TXB_2 synthesis than with nor-cantharidin, while PGI_2 is unaffected with both molecules. These results suggest that, being a strong inhibitor of protein phosphatase, cantharidin binds to cellular membranes, and the formation of a cantharidin-protein complex may interfere with normal membrane metabolism and activate the arachidonic cascade leading to an exaggerated production of vasoactive metabolites. These metabolites could be involved in cantharidin nephrotoxicity by disrupting homeostatic response. In contrast, by reducing PGI_2 and thromboxane release (which are suggestive of a lower inhibition of the constitutive COX-1), the nephrotoxic potential of nor-cantharidin is clearly lower than that of cantharidin.

Cantharidin treatment also increases GSH and oxidized GSH levels which is suggestive of oxidative stress. In addition, nor-cantharidin increases GSH content much less than cantharidin, with little variation of oxidized GSH content relative to the control cells. Taken together, our results suggest that nor-cantharidin prevents the unbalance of vasodilatator/vasconstrictor agents. However, the increase in GSH content could be conflicting with the increase in oxidized GSH levels, since it cannot be attributed to the oxidized GSH-Rd activity which is similar to that of the controls. Furthermore, the activity of GSH peroxidase which utilizes GSH during inactivation of the radicals was only increased at the highest dose of cantharidin and could not contribute to the elevation of GSH content. The increase in GSH could be explained either by an inhibition of its degradation or by the result of alterations in the membrane transport system. Whatever the mechanism involved, the in-

crease in GSH level probably provides information about the cellular oxidative events and a cantharidin-induced free radical generation can be suggested. In contrast, by increasing GSH and oxidized GSH levels much less than cantharidin, nor-cantharidin appears to induce lesser free radical generation.

In conclusion, a better understanding of the various mechanisms of cantharidin side effects can contribute to an easier comparison with nor-cantharidin effects. Our results indicate that (i) macrophages may also participate in cantharidin-induced vasoconstriction which could account for its higher deleterious effects, (ii) COX-2 could be an additional target of protein phosphatase inhibitors. Even if the mechanism of action of the two analogues seems to follow a similar pathway, nor-cantharidin appears to induce little side effects and lesser metabolic disturbances than cantharidin as shown by preventing hydroperoxide production and maintaining tissue homeostasis both in macrophages and in LLC-PK_1 cells. Finally, our results emphasize that cantharidin can be a useful tool to study the function of protein phosphatase in cell permeability and redox regulation. Because nor-cantharidin, which is routinely used as an anticancer drug in China, leads to little metabolic disturbances than cantharidin, it could be an attractive potential therapeutic in cancer chemotherapy in western countries.

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