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Protective effect of cyanidin 3-*O*-β-D-glucoside on ochratoxin A-mediated damage in the rat

Claudia Di Giacomo[1], Rosaria Acquaviva[1], Andrea Piva[2], Valeria Sorrenti[1], Luca Vanella[1], Gianfranco Piva[3], Gabriele Casadei[2], Luca La Fauci[4], Alberto Ritieni[5], Matteo Bognanno [4], Laura Di Renzo[6], Maria L. Barcellona[1], Mauro Morlacchini[7] and Fabio Galvano[4]*

[1]*Department of Biochemistry, Medical Chemistry and Molecular Biology, University of Catania, Vle A. Doria 6, 95100 Catania, Italy*
[2]*DIMORFIPA, University of Bologna, 40064 Ozzano Emilia, Bologna, Italy*
[3]*ISAN, Sacred Heart Catholic University of Piacenza, 29100 Piacenza, Italy*
[4]*STAFA Department, Mediterranean University of Reggio Calabria, P.zza S. Francesco 7, 89061 Reggio Calabria, Italy*
[5]*Department of Food Science, 'Federico II' University, 80055 Portici, Italy*
[6]*Department of Neuroscience, 'Tor Vergata' University, Rome, Italy*
[7]*CERZOO S. Bonico, 29100 Piacenza, Italy*

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The aim of the present study was to verify whether the oral administration of cyanidin 3-*O*-β-D-glucoside (C3G) might counteract damage induced by chronic exposure (28 d) to ochratoxin A (OTA) in rats and if its effect may be mediated by haeme oxygenase-1 (HO-1). Forty male Sprague–Dawley rats, individually caged, were divided into four groups of ten animals. A control group received a commercial diet, group C3G received the control diet supplemented with C3G (1 g/kg feed), group OTA received the control diet supplemented with 200 parts per billion of OTA, and group OTA + C3G received the OTA group diet supplemented with C3G (1 g/kg feed). After 4 weeks of treatment animals were killed and the liver, kidneys and brain of each rat were collected and homogenised to evaluate non-proteic thiol groups (RSH), lipid hydroperoxide (LOOH) levels, HO-1 expression and DNA fragmentation. Rats of the OTA group showed a significant ($P<0{\cdot}001$) decrease in RSH content of kidney and liver and a significant ($P<0{\cdot}001$) increase of LOOH in all the examined tissues compared with the control group. In the OTA + C3G group both RSH content and LOOH levels were similar to those observed in the control group, demonstrating that C3G was able to counteract the effects of OTA. A significant ($P<0{\cdot}001$) induction of HO-1 was evident in kidney and liver of both OTA and C3G groups. DNA damage occurred in all the examined tissues of the OTA group, whereas C3G was able to prevent it. The present study confirmed that the effects of OTA are mediated by oxidative stress and demonstrated that C3G efficiently counteracted deleterious effects of OTA because of its antioxidant and HO-1-inducing properties.

Cyanidin-3-*O*-β-glucoside: Ochratoxin A: Non-proteic thiol groups: Lipid hydroperoxides: Haeme oxygenase-1

Mycotoxin ochratoxin A (OTA) is produced as a secondary metabolite by certain *Penicillum* and *Aspergillum* fungal species and is a common contaminant of human foodstuffs and animal feed. OTA occurs in a variety of plant products and can enter the human food chain directly through cereals, grains, beans, spices and by food products such as coffee, grape juice, wine, beer and bread[1]. The carcinogenicity of OTA in rats and mice is well established. OTA induces renal tumours in rats of both sexes and in male mice[2,3]. In man, exposure to high levels of OTA in the diet has been suggested as a causative factor in 'Balkan endemic nephropathy' and in the development of urinary tract tumours[4–7], although a direct causal relationship is still under debate and the mechanism of OTA carcinogenicity is not known[1].

In a recent review by O'Brien & Dietrich[8] on its toxicological properties, OTA has justly been called 'the continuing enigma'. According to the authors, it is not yet clear whether the predominant toxic mechanism of OTA is of a genotoxic or epigenetic nature, such as induced cytotoxicity, oxidative cell stress or increased cell proliferation due to an imbalance between proliferative and antiproliferative intracellular signal pathways.

Both carcinogenicity and cytotoxicity of OTA have been related to free radical-mediated oxidative cell damage[9–14]. Schaaf *et al.*[10] attributed proximal tubule cell damage caused by OTA to the formation of reactive oxygen species such as the superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and peroxide ($ROO^{\cdot}$) which induce a wide range of lesions to cell components. Other authors reported that cytochrome P450 is able to stimulate

Abbreviations: C3G, cyanidin 3-*O*-β-D-glucoside; HO, haeme oxygenase; LOOH, lipid hydroperoxide; OTA, ochratoxin A; RSH, non-proteic thiol group.

* **Corresponding author:** Dr Fabio Galvano, fax 0965 322330, email fabio.galvano@unirc.it

OTA-dependent lipid peroxidation and that this action is mediated by OTA–$Fe^{3+/2+}$ complexes[15–17].

In view of these reports, it is conceivable that nutritional factors may affect the susceptibility to OTA's effects, since most of the natural antioxidants occurring in the diet can act both as free radical scavengers and as Fe chelators.

The list of natural compounds acting as antioxidants includes anthocyanins, a widespread group of water-soluble plant constituents collectively known as flavonoids. The anthocyanins' health properties are due to their particular chemical structure; their electron deficiency makes them very reactive towards reactive oxygen species. The most common anthocyanins in plants are cyanidins, largely present in the human diet through beans, fruits, vegetables and red wine. Cyanidin antioxidant and Fe-chelating properties have been demonstrated in numerous studies using several methods, both *in vivo* and *in vitro*[18,19], and a recent study carried out in our laboratory demonstrated that cyanidin 3-*O*-β-D-glucoside (C3G) is able to protect human fibroblasts against OTA-induced DNA damage[20].

The ability of several natural antioxidants to induce haeme oxygenase (HO)-1 expression has recently been reported[21–23]. HO is the rate-limiting enzyme in haeme degradation to carbon monoxide, Fe and bilirubin. To date, three isoforms of HO have been identified: HO-1, or inducible enzyme, HO-2 or constitutive isoform and HO-3[24–28].

HO-1, also known as stress protein HSP32, is present in normal conditions in the spleen[29]. Its expression can, however, increase in many tissues following stressful stimuli including heavy metals, heat shock, glutathione depletion, UVA radiation, endotoxin, hypoxia, hyperoxia, ischaemia–reperfusion and a wide range of conditions characterised by alteration of the cellular redox state[30–38]. A substantial body of evidence demonstrates that HO-1 induction represents an essential step in cellular adaptation to stress subsequent to pathological events[34,39–41]. Although HO-1 hyper-expression can be considered a marker of cellular stress, this enzyme can be also regarded as a potential therapeutic target in a variety of oxidant- and inflammatory-mediated diseases. In fact, it has recently been reported that naturally occurring antioxidants potently induce HO-1 expression, leading to an increased resistance to oxidative stress-mediated damage[22,23,42]. As a consequence, it has been hypothesised that the beneficial actions attributed to several natural substances could be due to their intrinsic ability to activate the HO-1 pathway[22,23,42].

The present *in vivo* study was performed to verify whether the chronic oral administration of C3G might counteract damage induced by chronic exposure to OTA in rats and if its effect may be mediated by HO-1.

Material and methods

Chemicals

OTA from *Aspergillus ochraceus* was purchased from Sigma-Aldrich (Milan, Italy) and C3G was purchased from Polyphenols Laboratories (Sandnes, Norway). The Qiamp DNA mini kit was purchased from Qiagen (Löhne, Germany). Monoclonal HO-1 antibody was from Stressgen Biotechnologies (Victoria, BC, Canada), anti-actin antibody was from Sigma Aldrich Co. (St Louis, MO, USA) and secondary horseradish peroxidase-conjugated anti-mouse antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence system for developing immunoblots and nitrocellulose membranes were purchased from Amersham Pharmacia Biotech (Milan, Italy). All other chemicals were purchased from Merck (Frankfurt, Germany).

Animals and treatment

The experiments reported in the present paper complied with current Italian law and met the guidelines of the Institutional Animal Care and Use Committee of Sacred Heart Catholic University of Piacenza (Italy). The experiments were performed in male Sprague–Dawley albino rats (150 g body weight and age 45 d at the beginning of experiments). They had free access to water and were kept at room temperature with a natural photo-period (12 h light–12 h dark cycle). Rats were subdivided into four groups (each group consisted of ten animals; each animal was placed into a separate cage) and received the test compounds orally, via their food pellets, for 4 weeks. A control group received a commercial balanced standard diet, group C3G received the standard control diet supplemented with C3G (1 g/kg feed), group OTA received the standard control diet supplemented with 200 parts per billion of OTA, group OTA + C3G received the standard diet of the OTA group supplemented with C3G (1 g/kg feed). Both OTA and C3G dosage were chosen accordingly to overall literature data relating to toxic chronic effect and antioxidant properties, respectively. After 4 weeks of daily treatment, animals underwent euthanasia by an overdose of anaesthetic (ethyl urethane, intraperitoneally) and the liver, kidneys and brain of each rat were rapidly removed in a cold room and immediately frozen (−80°C). Samples were processed within 1 week of collection.

Analytical procedures

Tissues were homogenised in 9 volumes of cold PBS. Samples of homogenate were used to evaluate non-proteic thiol groups (RSH) and lipid hydroperoxide (LOOH) levels, HO-1 expression and DNA fragmentation.

Lipid peroxidation

LOOH levels were measured in tissue homogenates following the oxidation of Fe^{+2} to Fe^{+3} in the presence of xylenol orange at $\lambda = 560$ nm[43]. The assay mixture contained, in a total volume of 1 ml: 100 μl homogenate, 100 μM-xylenol orange, 250 μM-ammonium ferrous sulfate, 90 % methanol, 4 mM-butylated hydroxytoluene and 25 mM-H_2SO_4. After 30 min incubation at room temperature, the absorbance at $\lambda = 560$ nm was measured using a U2000 Hitachi spectrophotometer (Hitachi, Ibaraki, Japan). Calibration was obtained using H_2O_2 (0·2–20 μM). Results are expressed as μmol/mg proteins.

Non-proteic thiol groups

Levels of RSH were measured, in 200 μl homogenate, using Hu's method[44] partially modified. This spectrophotometric assay is based on the reaction of thiol groups with

2,2-dithio-bis-nitrobenzoic acid at $\lambda = 412$ nm[44] in absolute ethanol ($\epsilon M = 13\,600$). Results are expressed as nmol/mg proteins.

Western blotting for haeme oxygenase-1 expression

Protein levels were visualised by immunoblotting with antibodies against HO-1. Briefly, samples of homogenates corresponding to 50 μg proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. In order to block non-specific binding sites, the membranes were incubated overnight with 5 % non-fat dry milk in 10 mM-tri(hydroxymethyl)-aminomethane-HCl (pH 7·4), 150 mM-NaCl, 0·05 % Tween 20 buffer at 4°C. After washing with the buffer, the membranes were incubated with a 1:1000 dilution of anti-HO-1 antibody for 1 h at room temperature under constant shaking. The filters were then washed and subsequently probed with horseradish peroxidase-conjugated anti-mouse IgG for HO-1 at a dilution of 1:2000. Chemiluminescence detection was performed using an enhanced chemiluminescence detection kit according to the manufacturer's instructions. Results were quantified and normalised to β-actin by using image analysis software (Scion Image; Scion Corp., Frederick, MD, USA).

Protein assay

Protein content was evaluated according to the method of Lowry *et al.*[45].

DNA damage

Genomic DNA was isolated from liver, kidney and brain homogenates using the Qiamp DNA micro kit (Qiagen) according to the manufacturer's instructions, electrophoresed on a 2 % agarose gel and stained by ethidium bromide[46]. The gels were then photographed under UV luminescence. Under these conditions, damaged DNA appears as a smear consisting of DNA fragments, whereas intact DNA has high molecular weight and does not migrate very far into the gel.

Statistical evaluation

Arithmetical means and standard deviations of all measured parameters were calculated. The results were analysed for statistical significance using ANOVA. A value of $P < 0{\cdot}001$ was taken as significant.

Results

During the experiment rats showed neither reduced vitality nor evident symptom of toxicity. No significant differences were observed as regards body weight and feed intake (data not shown) and no mortality was recorded. Fig. 1 reports the values of RSH content in kidney, liver and brain. In the OTA-treated group, a significant decrease in RSH content was observed in both kidney and liver, while values observed in brains of OTA-treated rats were similar to controls. According to the observed reduced levels of RSH, chronic administration of OTA led to a significant increase in LOOH levels (Fig. 2) in all the tissues under study.

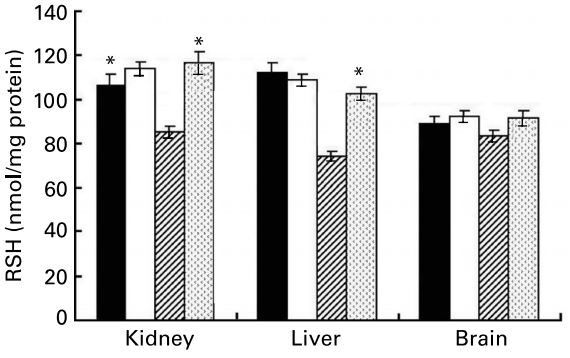

Fig. 1. Effect of chronic consumption of ochratoxin A (OTA; ■), cyanidin 3-*O*-β-D-glucoside (C3G; □) or OTA + C3G (▨) compared with control treatment (▤) on non-proteic thiol group (RSH) levels in rat kidney, liver and brain. Values are means of three determinations/sample (ten samples/group), with standard deviations represented by vertical bars. *Mean value was significantly different from that of the OTA group ($P < 0{\cdot}001$).

In animals fed on the diet supplemented with C3G, kidney and brain but not hepatic RSH contents were slightly higher than controls (Fig. 1). However, the same group of animals showed significantly reduced LOOH levels in all three tissues (Fig. 2), suggesting that the antioxidant effect of C3G is not merely due to its ability to preserve endogenous stores of RSH.

Chronic supplementation with C3G was also able to counteract the toxic effect of OTA. In fact, in the OTA + C3G group both RSH content and LOOH levels were similar to those observed in control rats (Figs. 1 and 2).

Fig. 3 reports results regarding HO-1 expression by Western blot analysis. A significant induction of HO-1 is evident in kidney and liver of both OTA and C3G groups; however, the most relevant HO-1 expression was observed in kidney of C3G-supplemented rats. The simultaneous intake of both substances caused an increase in the expression of HO-1 in liver but not in kidney when compared with controls. None of the dietary treatments significantly affected brain HO-1 expression.

The results obtained from DNA fragmentation indicated that following chronic consumption of OTA, DNA damage occurred in all three tissues examined (Fig. 4). Fig. 4 clearly shows the protective effect of C3G.

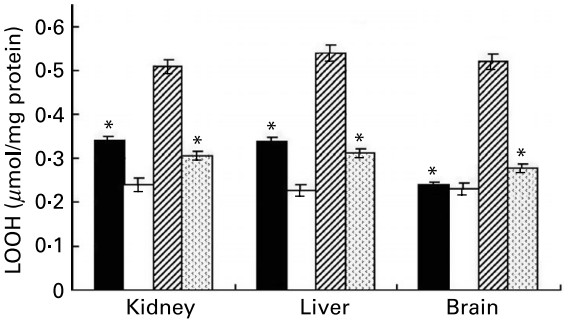

Fig. 2. Effect of chronic consumption of ochratoxin A (OTA; ■), cyanidin 3-*O*-β-D-glucoside (C3G; □) or OTA + C3G (▨) compared with control treatment (▤) on lipid hydroperoxide levels (LOOH) in rat kidney, liver and brain. Values are means of three determinations/sample (ten samples/group), with standard deviations represented by vertical bars. *Mean value was significantly different from that of the OTA group ($P < 0{\cdot}001$).

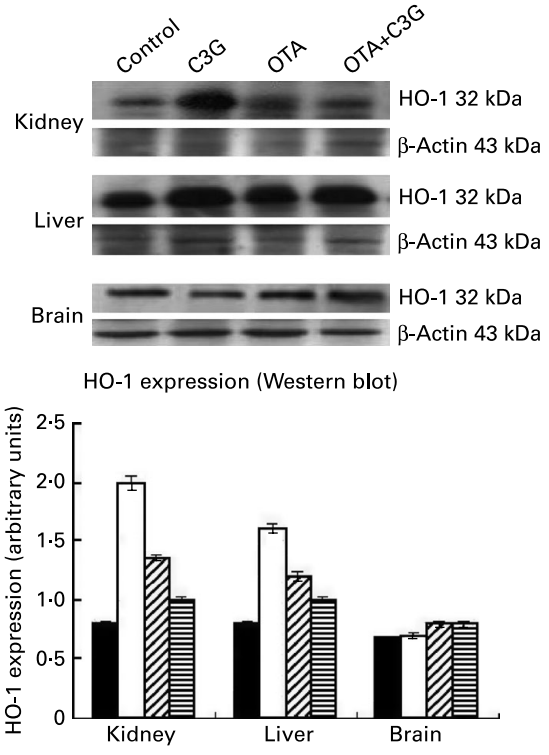

Fig. 3. Effect of chronic consumption of ochratoxin A (OTA; ■), cyanidin 3-*O*-β-D-glucoside (C3G; □) or OTA + C3G (▨) compared with control treatment (▭) on haeme oxygenase (HO)-1 protein expression in rat kidney, liver and brain. Values are means of ten samples/group, with standard deviations represented by vertical bars.

Discussion

It has been shown that oxidative damage contributes to the wide range of toxic effects of the mycotoxin ochratoxin[17,47–49]. The alterations in LOOH and RSH levels observed in the present study confirm the involvement of oxidative pathway in damage induced by OTA in kidney and liver. They also evidence the ability of the mycotoxin to affect brain tissue, as demonstrated by significantly increased LOOH levels and DNA damage found in brains of the OTA group. These results confirm that kidney is the target organ, but also demonstrate that OTA toxicity to other organs should not be underestimated.

Our working hypothesis was that natural antioxidants such as C3G might counteract OTA-induced damage. Data from these experiments demonstrated that chronic supplementation with C3G is able to efficiently counteract OTA-mediated oxidative changes. In fact, animals fed with a diet containing both OTA and C3G showed both RSH and LOOH levels similar to control values. In addition, the present results also suggest that the protective effects of C3G may be attributable not only to its antioxidant ability, but also to the HO-1-inducing property of the molecule. HO has both catabolic and anabolic roles in the cell. In the former capacity, it regulates cellular haeme and haemoprotein levels, including that of cytochrome P450[50]. In its anabolic role, HO produces bile pigments, carbon monoxide and Fe, all of which are biologically active: bile pigments function as antioxidants[51], carbon monoxide has recently been suggested to have a function in the generation of cGMP and in regulation of vascular tone[52–56], and Fe regulates the expression of various genes, including that of HO-1 itself[57].

In the present study, an increased expression of HO-1 was observed in liver and kidneys of C3G-supplemented rats. However, from data regarding HO-1 expression it is also evident that both OTA and C3G are able to induce the heat-shock protein 32 in rat liver and kidney, even if the maximal increase was observed in the C3G group. The ability of both molecules to induce HO-1 expression may be the result of different molecular actions: C3G could increase HO-1 expression due to its molecular structure. It has been reported, in fact, that plant-derived polyphenolic compounds possess chemical features required to trigger the induction of antioxidant and defensive genes[21,22]; then as reported for other polyphenols, C3G may activate the HO-1 gene via regulation of Nrf2 and/or the antioxidant-responsive element[23].

Although HO-1 hyper-expression may be considered a marker of cell stress, in view of the increasing evidence corroborating the importance of carbon monoxide and bilirubin (products of HO-1) in counteracting stress conditions, molecules with HO-1-inducing activity could also represent pharmacological strategies to increase cellular response and to increase cytoprotection. In fact, several reports showed the crucial participation of HO-1 gene expression in alleviating organ dysfunction and counteracting metabolic disorders in mammals, so this enzyme may be regarded as a potential target in a variety of oxidant-mediated diseases[35,36,38–40,50,51,58–62].

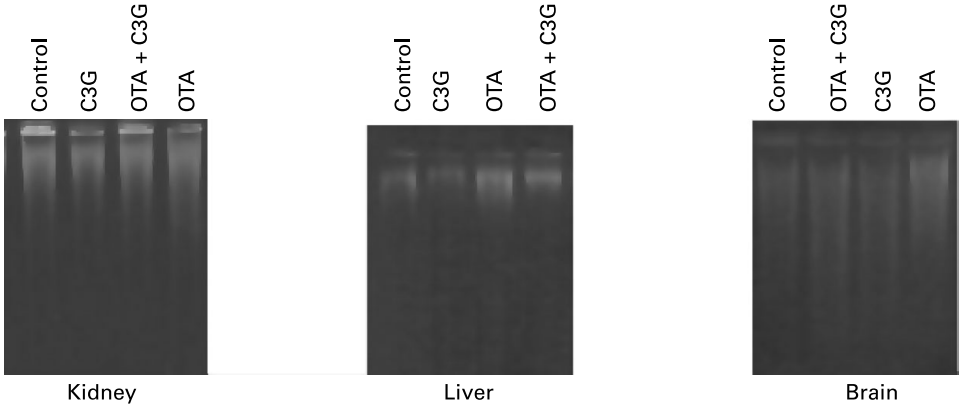

Fig. 4. DNA fragmentation in kidney, liver and brain of rats: effect of chronic oral consumption of ochratoxin A (OTA), cyanidin 3-*O*-β-D-glucoside (C3G) or OTA + C3G.

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Conversely, HO-1-inducing activity of OTA may be attributable to its RSH-decreasing effect. A correlation between HO and glutathione has in fact been documented, where HO-1 levels are augmented in conditions of glutathione depletion[31,33,63] and among exogenous chemical agents inducing HO-1, molecules that directly complex intracellular glutathione are included[38,64–66].

Data regarding the OTA + C3G group showed that hepatic and renal HO-1 expression is less pronounced compared with that observed in the OTA or C3G groups, but higher than in control. This result might be attributable to the capacity of chronic consumption of C3G to counteract RSH depletion induced by OTA. However, in view of the multiple response elements within HO-1 promoter and the multiplicity of interactions between components of the cell-signalling cascade[67] we cannot rule out the involvement of other molecular mechanisms mediated by the complex intracellular signalling network implicated in the induction of stress proteins.

The present study aimed to verify the hypothesis that chronic consumption of C3G might counteract OTA-mediated injury. The results reported here showed that chronic consumption of C3G, a naturally occurring antocyanin, potently induced liver and kidney HO-1 expression and increased RSH levels, leading to enhanced resistance to OTA-derived oxidative stress-mediated damage.

Coherently with results regarding brain RSH levels, none of the diet regimens induced significant changes in brain HO-1 expression even if a relevant increase in LOOH was observed in the OTA group. This result further supports the hypothesis that OTA-induced hyper-expression of HO-1 may be a consequence of decreased RSH levels. The lack of modification in brain HO-1 expression in C3G-supplemented rats may be not surprising in consideration of data in the literature reporting the tissue selectivity of several HO-inducers[57]; moreover, it has to be considered that in the brain the prominent form of HO protein is the HO-2 isoform[68,69], whose transcription levels are sensitive to cellular redox state[70,71].

Analysis of DNA fragmentation evidenced that, following chronic consumption of OTA, DNA damage occurred in all three tissues under examination (liver, kidney and brain). These results may support the hypothesis that OTA toxicity is not limited to organs dedicated to its metabolism and excretion. At the present we cannot discriminate the type of mechanism involved (apoptotic or necrotic); however, based on results of experiments in progress in our laboratory, we are inclined to deem that DNA damage observed in the OTA group is apoptotic (F. Galvano, R. Acquaviva, L. La Fauci, C. Di Giacomo, unpublished results). In addition, our data indicated a clear DNA protection exerted by C3G, in agreement with previous reports by Acquaviva *et al.*[19] demonstrating a protective effect of C3G on DNA cleavage. Overall, the results obtained demonstrate that C3G is able to counteract the deleterious effects of chronic consumption of OTA and provide evidence that its nutritional supplementation may contribute in preventing OTA-induced damage.

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