The Possible Chemosensitizing Effect of Different Doses of Indol-3-Carbinol on Transplantable Tumor Model Treated with Doxorubicin

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Abstract. Background: Ehrlich carcinoma is a transplantable tumor model used frequently in cancer studies. Doxorubicin (DOX) is one of the anthracyclines that is frequently used in treatment of various types of malignancies including breast, prostate and lung cancer. Indole-3-carbinol (I3C) is a phytochemical that was suggested to have potent anti-tumor and chemosensitizing effects. Objective: To detect the possible chemosensitizing effects of different doses of I3C on solid Ehrlich carcinoma (SEC) treated with DOX in mice. Materials and methods: One hundred and forty mice were divided into seven equal groups as follows: Control untreated group, solid Ehrlich carcinoma (SEC), SEC + DOX, SEC + I3C 1000 ppm, SEC + I3C 2000 ppm, SEC + DOX + I3C 1000 ppm and SEC + DOX + I3C 2000 ppm. Tumor volume, survival rate, tissue glutathione reductase (GR), tissue glutathione peroxidase (GPx), tissue tumor necrosis factor alpha (TNF-α) and tissue interleukin-6 (IL-6) were determined. Parts of the tumor were subjected to histopathological and immunohistochemical examination. Results: DOX and/or I3C produced significant increase in the survival rate, tissue GPx and tissue GR with significant decrease in tumor volume, tissue TNF-α and tissue IL-6 compared to SEC group. Moreover, they improved the histopathological changes with significant increase in tissue caspase-3 activity and p53 compared to SEC group. These effects were significant in DOX/I3C combination groups compared to the use of each of these drugs alone. Conclusion: I3C- in a dose dependent manner - had a chemosensitizing effect against transplantable tumor model treated with DOX in mice and this might represent an adjuvant to the traditional drugs used in cancer chemotherapy.

INTRODUCTION

The use of the traditional anticancer agents such as 5-fluorouracil, methotrexate, doxorubicin (DOX) and cisplatin was faced by their harmful adverse effects [1]. In an attempt to increase the sensitivity of various types of malignancies to the traditional anticancer agents and thereby decreasing the effective chemotherapeutic dose and adverse effects, various approaches were investigated. One of them is the search for natural compounds with anticancer properties that can be used in combination with the traditional anticancer agents [2]. Epidemiological studies have suggested an important link between dietary intake of cruciferous vegetables and decreased risk of cancer development. Indole-3-carbinol (I3C) is one of these substances that had been shown to suppress the proliferation of various types of cancers including breast, colon, stomach, prostate and endometrial cancer [3]. I3C was thought to act by targeting signaling pathways that control cell cycle, hormonal homeostasis and cell proliferation [4]. Moreover, I3C was proven to inhibit all types of tumorigenesis in different types of tissues including mammary glands, liver, lung, cervix, and gastrointestinal tract in different animal models.
I3C was shown to suppress the growth of breast cancer cells of estrogen receptor positive and estrogen receptor negative by inhibiting cyclin-dependent kinase 6 (CDK6), inducing p27 expression and reducing the level of retinoblastoma protein. Other studies indicated that I3C also induced cell cycle arrest in breast cancer cells and inhibited CdK6 [5]. Moreover, I3C was shown to suppress the growth of human prostate cancer cells in a dose- and time dependent manner by repressing the expression of androgen receptors [6]. Also, I3C was known to induce estradiol 2-hydroxylase and reduce estrogen activity, thereby inhibiting spontaneous occurrence of endometrial adenocarcinoma in females. These studies throw a light on the value of I3C in cancer prevention and therapy [7]. These findings made I3C a rich media for use in human trials in various types of cancers including breast cancer, cervical carcinoma, vulvar intraepithelial neoplasia and respiratory papillomatosis [8]. The aim of this study was to detect the possible chemosensitizing effects of different doses of I3C on solid Ehrlich carcinoma (SEC) treated with DOX in mice.

MATERIALS AND METHODS

Drugs used. Doxorubicin (DOX) was commercially available in powder form for injection purchased from Carlo Erba, Turkey. It was dissolved in normal saline and administered by intraperitoneal injection in a dose of 4 mg/kg body weight once weekly for 4 weeks. Indole-3-carbinol (I3C) was purchased from Sigma Aldrich Co. and administered daily orally in diet.

Solid Ehrlich Carcinoma (SEC) tumor model. A model of SEC was used, where 1X10⁶ of the Ehrlich carcinoma cells (ECC) obtained from the oncology unit of the department of biology, faculty of science, Tanta university, Egypt were implanted subcutaneously into the right thigh of the hind limb of mice. A solid tumor mass (about 100 mm³) was developed within 12 days [9].

Classification of animals. In this study, we used one hundred and forty BALB/c mice weighing about 18–25 grams. All the experiments were conducted according to the National Research Council’s guidelines. Animal handling was followed according to Helsinki declaration of animal ethics. The animals were divided into seven equal groups of twenty mice each as follows:

Group (1): is the normal control group, received intraperitoneal injection of normal saline once weekly for 4 weeks.

Group (2): Ehrlich tumor cells were implanted subcutaneously into the right thigh of the hind limb of mice [9].

Group (3): DOX was given by intraperitoneal injection on days 0, 7, 14, 21 after subcutaneous implantation of Ehrlich tumor cells [10].

Group (4): Mice were put on diet containing 1000 ppm I3C one week before and continued for 6 weeks after subcutaneous implantation of Ehrlich tumor cells [11].

Group (5): Mice were put on diet containing 2000 ppm I3C one week before and continued for 6 weeks after subcutaneous implantation of Ehrlich tumor cells [12].

Group (6): Mice were put on diet containing 1000 ppm I3C one week before and continued for 6 weeks after implantation of Ehrlich tumor cells concomitantly with intraperitoneal injection of DOX on days 0, 7, 14, 21 after subcutaneous implantation of Ehrlich tumor cells.

Group (7): Mice were put on diet containing 2000 ppm I3C one week before and continued for 6 weeks after subcutaneous implantation of Ehrlich tumor cells concomitantly with intraperitoneal injection of DOX on days 0, 7, 14, 21 after subcutaneous implantation of Ehrlich tumor cells.

Assessment of the time-course effects of different treatments on tumor volume of SEC. Tumor volumes were recorded from the start point at 15th day post-implantation and thereafter every 5 days till the last record at the 40th day post-implantation prior to scarification of the survived mice using a Vernier caliper (Tricane Brand, Shanghai, China). Tumor volume (V) was calculated as V (mm³) = (a² × b)/2, where a (small diameter), and b (large diameter) are perpendicular, expressed in millimeters (mm).

Recording of the survival rate. The day of implantation of ECC was considered zero point of the experiment for recording and analysis of the survival rate weekly for 6 weeks (by recording number of the survived mice in each group at the end of each week).
At the end of the study, all mice were sacrificed. The tumor was excised and divided into two parts; one for homogenization and the other for histopathological and immunohistochemical examination. The tumor was homogenized for determination of tissue glutathione reductase (GR) activity according to the method of Manso and Wroblewski [14], tissue tumor necrosis factor-alpha (TNF-α) using mouse TNF-α ELISA kits supplied by RayBiotech, Inc. according to the instructions of the manufacturer and tissue interleukin 6 (IL-6) using mouse IL-6 ELISA kits supplied by RayBiotech, Inc. according to the instructions of the manufacturer. Tissue glutathione peroxidase (GPx) was determined in the supernatant using BIOXYTECH GPx-340TM assay kit produced by OXIS International, Inc., USA. The GPx assay was based on the oxidation of NADPH to NADP⁺, which is accompanied by a decrease in absorbance at 340 nm [15].

**Histopathological and immunohistochemical examination.** The SEC sections were prepared and stained with hematoxylin and eosin (H&E) and examined under light microscope. Assessment of tumor tissue p53 was carried out in formaline-fixed, paraffin embedded SEC sections using Zymed’s 2nd generation kit that utilizes the labeled streptavidin–biotin staining methodology (Zymed Laboratories Inc., Carlton Court, south San Francisco, USA). Positive nuclei for p53 accumulation stained brown. The tumor was considered to be p53-positive if more than 10% of cells showed positive staining. The number of cells showing nuclear accumulation of p53 in positive tumors was expressed as follows: (+++): the largest number of cells showing positive nuclear staining for p53; (+++): intermediate number of p53-positive cells; (++): indicates lower number of cells with p53-stained nuclei [16].

Immunohistochemistry for caspase-3 was performed in sections prepared from formalin-fixed, paraffin-embedded tissue using the avidin–biotin immunodetection complex method according to manufacturer’s instruction (Labvision, USA). Interpretation of results was done semiquantitatively by evaluating the intensity and distribution of positive cells. The intensity of caspase-3 immunostaining was assessed as follows: none = 0, mild = 1, moderate = 2 and strong = 3. The immunohistochemical histological score (H-score) was then calculated by multiplying the intensity by the percentage of tumor cells showing positive staining for caspase-3, creating a range of possible scores of 0–300 [17,18].

**Statistical analysis.** The data obtained were subjected to one way ANOVA and Tukey's multiple comparison test. Data were presented as mean ± S.E.M. Differences between the means of different groups were considered significant at a level of p-value less than 0.05

**RESULTS**

**Effect of different treatments on tumor volume.** Administration of DOX and/or I3C to mice resulted in significant decrease in tumor volume compared to SEC group. The decrease in tumor volume was significant in the groups that received DOX/I3C combination compared to the groups that received either DOX or I3C alone. The decrease in tumor volume was significant in the group that received DOX/2000 ppm I3C combination compared to the group that received DOX/1000 ppm I3C combination (Fig. 1).
**Fig. 1:** The effect of different treatments on tumor volume (mm$^3$)

- * Significant compared to the control group
- # Significant compared to SEC group
- + Significant compared to SEC+DOX group
- ^ Significant compared to SEC+I3C 1000 group
-  Significant compared to SEC+I3C 2000 group
- $ Significant compared to SEC+DOX+I3C 1000 group

**Effect of different treatments on the survival rate.** Subcutaneous implantation of Ehrlich carcinoma cells (ECC) resulted in significant decrease in the survival rate compared to the control untreated group. Administration of DOX and/or I3C to mice resulted in significant increase in the survival rate compared to SEC group. The increase in the survival rate was significant in the groups that received DOX/I3C combination compared to the groups that received either DOX or I3C alone. The increase in the survival rate was significant in the group that received DOX/2000 ppm I3C combination compared to the group that received DOX/1000 ppm I3C combination (Table 1).

**Table 1:** Comparative statistics for survival rate in the studied groups at the end point of the experiment (The 42$^{nd}$ day post-implantation)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Survival rate (%)</th>
<th>Survival duration (weeks; % confidence interval (CI), lower bound to upper bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100%</td>
<td>(6.0 ± 0.0 weeks; 95% CI, 6.00–6.00)</td>
</tr>
<tr>
<td>SEC</td>
<td>50%$^a$</td>
<td>(5.2 ± 0.3 weeks; 95% CI, 4.73–5.93)</td>
</tr>
<tr>
<td>SEC + DOX</td>
<td>75%$^b$</td>
<td>(5.8 ± 0.1 weeks; 95% CI, 5.67–6.12)</td>
</tr>
<tr>
<td>SEC + I3C 1000</td>
<td>60%$^b$</td>
<td>(5.4 ± 0.3 weeks; 95% CI, 4.73–6.08)</td>
</tr>
<tr>
<td>SEC + I3C 2000</td>
<td>70%$^b$</td>
<td>(5.7 ± 0.1 weeks; 95% CI, 5.43–6.03)</td>
</tr>
<tr>
<td>SEC + DOX + I3C 1000</td>
<td>85%$^{bced}$</td>
<td>(5.9 ± 0.1 weeks; 95% CI, 5.85–6.1)</td>
</tr>
<tr>
<td>SEC + DOX + I3C 2000</td>
<td>100%$^{bdef}$</td>
<td>(6.0 ± 0.0 weeks; 95% CI, 6.00–6.00)</td>
</tr>
</tbody>
</table>

- $^a$ Significant compared to the control group
- $^b$ Significant compared to SEC group
- $^c$ Significant compared to SEC+DOX group
- $^d$ Significant compared to SEC+I3C 1000 group
- $^e$ Significant compared to SEC+I3C 2000 group
- $^f$ Significant compared to SEC+DOX+I3C 1000 group

**Effect of different treatments on the antioxidant status.** Subcutaneous implantation of ECC resulted in significant decrease in tissue GPx and GR activity compared to the control untreated group. Administration of DOX and/or I3C to mice resulted in significant increase in tissue GPx and GR activity compared to SEC group. The improvement in the antioxidant status was
significant in the groups that received DOX/I3C combination compared to the groups that received either DOX or I3C alone. This improvement was significant in the group that received DOX/2000 ppm I3C combination compared to the group that received DOX/1000 ppm I3C combination (Table 2).

**Effect of different doses of I3C on tissue TNF-α and IL-6.** Subcutaneous implantation of ECC resulted in significant increase in tissue TNF-α and IL-6 compared to the control untreated group. Administration of DOX and/or I3C to mice resulted in significant decrease in tissue TNF-α and IL-6 compared to SEC group. This decrease was significant in the groups that received DOX/I3C combination compared to the groups that received either DOX or I3C alone. This decrease was significant in the group that received DOX/2000 ppm I3C combination compared to the group that received DOX/1000 ppm I3C combination (Table 2).

**Table 2:** Effect of different treatments on tumor tissue GPx, GR, TNF-α and IL-6 in the studied groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SEC</th>
<th>DOX + SEC</th>
<th>I3C 1000 + SEC</th>
<th>I3C 2000 + SEC</th>
<th>DOX + I3C 1000+ SEC</th>
<th>DOX + I3C 2000+ SEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue GPx (U/g tissue)</td>
<td>1.44±0.02</td>
<td>0.66±0.02</td>
<td>1.02±0.04</td>
<td>0.82±0.03</td>
<td>0.93±0.02</td>
<td>1.17±0.02</td>
<td>1.3±0.04</td>
</tr>
<tr>
<td>Tissue GR (U/g wet tissue/min)</td>
<td>848.3±10.4</td>
<td>440.4±8.4</td>
<td>642.4±12.3</td>
<td>559.2±8.1</td>
<td>586.8±8.7</td>
<td>743.5±9.2</td>
<td>807.1±13.0</td>
</tr>
<tr>
<td>Tissue TNF-α (pg/g tissue)</td>
<td>216.01±6.75</td>
<td>1320.5±22.5</td>
<td>813.65±9.03</td>
<td>1031.1±24.6</td>
<td>918.65±9.76</td>
<td>704.7±16.2</td>
<td>492.9±22.8</td>
</tr>
<tr>
<td>Tissue IL-6 (pg/g tissue)</td>
<td>208.01±6.3</td>
<td>1421.5±20.5</td>
<td>821.6±8.4</td>
<td>1124.2±12.5</td>
<td>932.6±10.1</td>
<td>763.45±9.86</td>
<td>541.7±8.8</td>
</tr>
</tbody>
</table>

a Significant compared to the control group  
b Significant compared to SEC group  
c Significant compared to SEC+DOX group  
d Significant compared to SEC+I3C 1000 group  
e Significant compared to SEC+I3C 2000 group  
f Significant compared to SEC+DOX+I3C 1000 group

**Histopathological and immunohistochemical findings.** Subcutaneous implantation of ECC resulted in development of Ehrlich solid tumor showing sheets of small, higher chromatophilic tumor cells of variable shape representing cell proliferation regions surrounding areas of necrosis and differentiated cells (Fig. 2a) with negative staining for p53 (Fig. 3a) and significant decrease in the expression of caspase 3 (Fig.4a) compared to the control group. Administration of DOX to mice resulted in improvement of the histopathological picture manifested as sheets of focal necrosis and apoptosis (Fig. 2b) with positive p53 expression (Fig. 3b) and significant increase in the expression of caspase 3 (Fig. 4b) compared to SEC group. Administration of I3C resulted in improvement of the histopathological picture in a dose-dependent manner manifested as sheets of malignant cells with focal necrosis and apoptosis (Fig. 2c,d) with positive p53 expression (Fig. 3c,d) and significant increase in the expression of caspase 3 (Fig. 4c,d). Administration of DOX/I3C combination resulted in improvement of the histopathological picture in a dose-dependent manner manifested as extensive necrosis (Fig. 2e,f) with positive p53 expression (Fig. 3e,f) and significant increase in the expression of caspase 3 (Fig. 4e,f) compared to the use of each of these drugs alone.
Fig. 2: A photomicrograph of a) SEC sections from mice showing sheets of small, higher chromatophilic malignant cells of variable shape representing cell proliferation surrounding small areas of necrosis; b) SEC sections from mice that received DOX showing moderate necrosis; c) SEC sections from mice that received I3C 1000 ppm showing collections of malignant cells with focal necrosis; d) SEC sections from mice that received I3C 2000 ppm showing focal necrosis; e) SEC sections from mice received DOX/I3C 1000 ppm showing extensive necrosis with small collections of malignant cells; f) SEC sections from mice received DOX/I3C 2000 ppm showing extensive necrosis.
Fig. 3: A photomicrograph of p53 staining of a) SEC sections from mice showing negative staining for p53; b) SEC sections from mice that received DOX showing positive (+++) p53 expression; c) SEC sections from mice that received I3C 1000 ppm showing positive (+++) p53 expression; d) SEC sections from mice that received I3C 2000 ppm showing positive (+++) p53 expression; e) SEC sections from mice that received DOX/I3C 1000 ppm combination showing positive (++++) p53 expression; f) SEC sections from mice that received DOX/I3C 2000 ppm combination showing positive (++++) p53 expression (PAP X 250).
Fig. 4: A photomicrograph of immunohistochemical staining of caspase-3 in a) SEC group showing faint immunostaining for caspase-3 in 5% of tumor tissue (H-score = 5); b) DOX-treated group showing moderate positive staining for caspase-3 in 30% of tumor tissue (H-score = 60); c) I3C 1000 ppm-treated group showing mild positive staining for caspase-3 in 20% of tumor tissue (H-score = 20); d) I3C 2000 ppm-treated group showing mild positive staining for caspase-3 in 30% of tumor tissue (H-score = 30); e) DOX/I3C 1000 ppm combination group showing moderate positive staining for caspase-3 in 55% of tumor tissue (H-score=110); f) DOX/I3C 2000 ppm combination group showing strong positive staining for caspase-3 in 70% of tumor tissue (H-score=210).

DISCUSSION

Cancer is a group of diseases associated with unregulated cell growth. Ehrlich carcinoma is an undifferentiated transplantable carcinoma that has rapid proliferation rate, 100% malignancy and not has tumor-specific antigen. Due to these properties, Ehrlich carcinoma represented an important transplantable tumor model that is used in cancer studies [19].
In the present study, subcutaneous implantation of ECC resulted in significant decrease in the survival rate, tissue GPx and GR activity compared to the control group. These results were in agreement with Kabel et al. [20] and Metwally et al. [21]. Oxidative stress was involved in cellular processes including apoptosis, DNA damage, cellular proliferation and carcinogenesis. It was reported that GPx activity decreased in Ehrlich tumor–bearing mice which was associated with decreased production of NADPH [21].

In the present study, implantation of ECC resulted in significant increase in tumor tissue TNF-α and IL-6 compared to the control group which was in the same line with other studies which reported that TNF-α and IL-6 have an important role in cancer development [22]. TNF-α and IL-6 are immunomodulatory cytokines that are frequently expressed in various types of cancer. The presence of TNF-α and IL-6 in advanced metastases and the positive correlation between TNF-α and IL-6 levels and progression of cancer indicates a critical role of TNF-α and IL-6 in the tumor microenvironment [23]. Moreover, TNF-α and IL-6 can affect tumor cell proliferation and survival through its effect on the genes encoding nuclear factor-kB–dependent antiapoptotic molecules. Also, TNF-α and IL-6 were found to promote the development of distant metastasis and cancer cachexia [24].

Caspase 3 is a protein that interacts with caspase-8 and caspase-9. Activation of these caspses plays a central role in the execution-phase of apoptosis. Subcutaneous implantation of ECC resulted in significant decrease in caspase 3 activity compared to the control group which was in the same line with other studies that reported that injection of ECC resulted in marked inhibition of apoptosis manifested by significant decrease of caspase 3 activity [25].

Tumor protein p53, also known as p53, is a protein encoded by homologous genes in various organisms which was proven to prevent cancer formation. P53 gene was found to be the most frequently mutated gene (more than 50%) in human cancer, indicating its vital role in preventing cancer formation. P53 gene encodes proteins that bind to DNA and regulate gene expression to prevent gene mutations [26]. This was confirmed by the results of the present study where subcutaneous implantation of ECC resulted in significant decrease in the expression of p53 which had a direct effect on tumor progression and invasiveness.

In the present study, Administration of DOX resulted in significant improvement in the survival rate and the antioxidant parameters with significant decrease in tumor volume, tissue TNF-α and IL-6 and alleviated the histopathological changes with significant increase in p53 expression and caspase 3 activity compared to SEC group. These results were in agreement with El-Dayem et al. [27] and Osman et al. [28] who reported that DOX has potent antioxidant and anti-inflammatory properties which, with its effects on apoptosis, may contribute to its anti-tumor properties.

It was reported that DOX might affect apoptosis through enhancing the expression and activity of caspases [29]. Also, Wang et al. [30] suggested that DOX may regulate cell differentiation through inhibition of Ras signaling. Takahashi [31] suggested that DOX suppressed TNF-alpha induced generation of free radicals and so has protective effects against tissue damage induced by reactive oxygen species which was in the same line with the results of the present study. On the other hand, Yang et al. [32] suggested that the anti-tumor properties of DOX on cancer cells are due to generation of free radicals leading to damage of the cellular membranes, DNA destruction, oxidative stress, and induction of apoptosis.

Indole-3-carbinol (I3C) is a phytochemical that is found in large amounts in cruciferous vegetables and is considered as an eminent chemopreventive agent that has antimitagenic, antitumorigenic, and antiestrogenic properties in experimental studies. In the present study, I3C, in a dose-dependent manner, resulted in significant improvement in the survival rate and the antioxidant parameters with significant decrease in tumor volume, tissue TNF-α and IL-6 and alleviated the histopathological changes with significant increase in p53 expression and caspase 3 activity compared to SEC group. These results were in agreement with Arora et al. [33] and Wang et al. [4] who reported that the antitumor effect of I3C was attributed to its antioxidant, anti-inflammatory, antiproliferative and apoptosis inducing properties.
Mao et al. [34] reported that I3C induces apoptosis and slows tumor cell growth in vivo and in vitro. I3C was reported to have anti-inflammatory effects by inhibiting production of the proinflammatory cytokines, thereby inhibiting the expression of nuclear factor-kB and decreasing expression of TNF-alpha and inducible nitric oxide synthase. Moreover, Acharya et al. [35] found that I3C therapy has potent antioxidant effects which may contribute to its exceptional anti-cancer properties. Also, Arora et al. [33] reported that I3C decreases the expression of p-glycoprotein which is responsible for tumor resistance to chemotherapeutic agents such as methotrexate, cisplatin and DOX. So, I3C can be combined with chemotherapeutic agents to potentiate their anti-tumor effect and decrease resistance of cancer cells.

Synergistic interactions were found between I3C and the traditional chemotherapeutic agents such as cisplatin, methotrexate and DOX. The combination of I3C and DOX had the ability to kill tumor cells in vitro and in vivo. Also, it was found that indole-3-carbinol cyclic tetrameric derivative CTet synergizes with cisplatin and DOX in breast cancer cell lines [36]. This was in the same line with the results of the present study where I3C in a dose-dependent manner, in combination with DOX, was able to produce significant improvement in the survival rate and the antioxidant parameters with significant decrease in tumor volume and the pro-inflammatory cytokines and alleviated the histopathological changes with significant increase in p53 expression and caspase 3 activity compared to use of each of these drugs alone.

CONCLUSIONS

I3C- in a dose dependent manner - had a chemosensitizing effect to DOX against transplantable tumor model in mice. These effects can be attributed to the anti-proliferative, antioxidant and anti-inflammatory properties of I3C together with its ability to induce apoptosis in cancer cells. This might represent a new adjuvant line of treatment to the traditional drugs used in cancer chemotherapy.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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