Enhanced Therapeutic Effect of Cytidine-5′-Diphosphate Choline when Associated with G_{M1} Containing Small Liposomes as Demonstrated in a Rat Ischemia Model

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Purpose. Cytidine-5′-diphosphate choline (CDPc) was encapsulated in long-circulating unilamellar vesicles (SUVs) to improve the drug's biological effectiveness.

Methods. SUVs made up of diaplmitoylphosphatidylcholine/diaplmitoylphosphatidylserine/cholesterol (7:4:7 molar ratio) and 8 mol % of ganglioside G_{M_1} were prepared by extrusion through polycarbonate filters (mean diameter 50 nm). The formulation effectiveness was evaluated by an *in vivo* model of cerebral ischemia on Wistar rats.

Results. The enhanced delivery of CDPc into the brain improved the therapeutic effectiveness of the drug. CDPc-loaded SUVs improved the survival rate of ischemized and reperfused Wistar rats (320-350 g) by ~66% compared with the free drug. Liposome formulation was also able to effectively protect the brain against peroxidative damage caused by post-ischemic reperfusion. SUVs lowered the conjugated diene levels of the cerebral cortex. The liposomal delivery system did not alter the distribution patterns in the various cerebral lipid fractions of the drug, radiolabeled with ^{14}C-CDPc.

Conclusions. CDPc-loaded SUVs were able to protect the brain against damage induced by ischemia. A possible clinical application is envisaged.

KEY WORDS: CDP-choline; long-circulating liposomes; ischemic injury; biological effectiveness.

INTRODUCTION

Delivery of hydrophilic drugs to the mammalian brain is restricted by the blood-brain barrier (BBB). This barrier, which hampers the free movement of many substances from the blood to the brain (1), is due in part to the tight intercellular junctions between brain capillary endothelial cells (2). It is suggested that some drug delivery systems, e.g. microspheres and liposomes, can improve drug delivery to the brain across the BBB (3). Liposomes containing cerebrotonic and neurotrophic drugs, i.e. cytidine-5′-diphosphate choline (CDPc), may be of great interest in the treatment of diseases concerning the central nervous system.

CDPc is a therapeutic agent widely used in the treatment of Parkinsonism, extrapyramidal diseases and consciousness disorders in brain injury. CDPc contributes to the repair of the membranous structures of brain cells that have been broken down by cerebrovascular damage (4). Unfortunately, the limiting factors in CDPc therapy are poor ability to pass through the BBB, due to its polar nature, and the small amounts, about 0.25% of the total administered dose, that reach the active site (5). A possible strategy in order to increase the quantity of CDPc in the central nervous system could be incorporation into liposomes.

Previous studies (6-8) showed that CDPc is entrapped in the aqueous phase of the liposome structure. Therefore, the volume of the entrapped aqueous phase is of great importance in the realization of a delivery device with a high encapsulation efficiency (9). Recently, *in vivo* experiments (10) have shown the effectiveness of CDPc-liposome formulations in the treatment of ischemized rats.

The aim of our study was to improve the liposome formulation parameters, i.e., the CDPc encapsulation efficiency, liposome mean size and size distribution. The formulation of 50 nm vesicles may ensure the escape of CDPc-loaded liposome through BBB fenestration resulting from pathological conditions. The biological effectiveness was assayed by means of an experimental *in vivo* model of partial cerebral ischemia. The enhanced delivery of CDPc to the brain was monitored by means of ^{14}C-CDPc.

MATERIALS AND METHODS

Chemicals

Dipalmitoyl-DL-α-phosphatidyl-L-serine (DPPS) and cholesterol (CH) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (DPPC) was purchased from Fluka Chemicals Co. (Buchs, Switzerland). Ganglioside G_{M1} was produced by Boehringer Mannheim. Before each experiment, the phospholipid purity (>99%) was controlled by two-dimensional TLC on silica gel plates (10). CDPc (purity 99.5%) was kindly provided by Cyanamid-Italy. Cytidine-5′-diphospho-[*methyl-^{14}C*] choline (50-60 mCi/mmol) was purchased from Amersham International plc (Buckinghamshire, UK). Double distilled pyrogen-free water was used. All other materials and solvents were of analytical grade.

Liposome Preparation

The aqueous drug solution and the aqueous dispersions of multilamellar vesicles (MLVs) were prepared as previously reported (10). Lipid concentrations of 50 mg · ml^{-1} were employed.

The frozen and thawed MLVs were obtained by freezing the MLVs in liquid nitrogen and thawing the samples at 50°C. Eight cycles of freeze and thaw were carried out. The resulting MLVs were extruded by means of a stainless steel extrusion device (Lipex Biomembranes, Vancouver, B.C.) equipped with 10-ml water-jacketed "thermobarrel" connected to a thermostat, which allowed extrusion at 50°C. Extrusion was carried out through two (stacked) polycar-

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bonate filters (25 nm diameter; Nucleopore Corp., Pleasanton, California, USA) at a nitrogen pressure of up to 5600 kPa. To achieve small unilamellar vesicles (SUVs), the extrusion procedure consisted of ten passages of the MLVs through 400 nm filters, followed by other cycles of ten passages through 200 nm and 50 nm polycarbonate filters. The liposomal suspension (DPPC/DPPS/CH 7:4:7 molar ratio) contained 8 mol % of ganglioside G_{M1}.

Entrapment and Release of CDPc

To evaluate the loading capacity, free CDPc was removed from the SUV suspension by a gel-filtration technique (10). The amount of CDPc encapsulated in the liposomes was determined by HPLC (8), destroying the phospholipid bilayer structures with a mixture of methylene chloride/methanol (2:1 v/v) (10). The amount of CDPc entrapped in the liposomes was expressed as encapsulation capacity (EC) (mL/mmol) and drug content (DC) (percentage of drug in the liposome formulation) as reported elsewhere (11). The drug release from liposomes was carried out by dialysis of the SUV suspension with a Spectrapor/por 2 membrane MWCO 12-14000 (Spectrum, Medical Industries, Inc., Los Angeles, California). The release experiments were carried out at 37°C (10).

Sizing of Liposomes

Photon correlation spectroscopy (PCS) was used to determine the vesicle size. The apparatus consisted of an Argon ion laser (Spectra Physics mod. 166), equipped with a sample holding system PC 8 Malvern (Worcestershire, UK), a microcontrol precise mechanical goniometer and an optical system Melles-Griot f. 150. The intensity of the scattered light was detected at 90° to the incident beam with an RCA (Fishers, New York, USA) mod. C 31034 photomultiplier cooled to −30°C. Correlation data were analyzed by the standard cumulant method to determine the average radius and the polydispesity index.

^{31}P-Nuclear Magnetic Resonance

^{31}P-NMR was employed to provide an indication of the lamellarity of the various liposome samples. Briefly, Mn^{2+} was added to the vesicle colloidal suspension (2 ml, 50 μmol phospholipid per ml in a 10 mm diameter NMR tube) at levels high enough (5 mM) to eliminate the ^{31}P-NMR signal arising from those phospholipids facing the external medium. Proton-decoupled ^{31}P-NMR spectra were performed on a GN500 MHz spectrometer operating at 202.45 MHz. Trimethyl phosphate (Aldrich) (10% in D_2O) was used as a reference and set at 0.0 ppm. The number of scans was 9000 with a spectral width of 20 kHz and 8 k data points. Ninety degree pulses were used with a pulse delay of 250 ms and an acquisition time of 205 ms. The average number of bilayers ⟨N⟩ was calculated from the following expression (12):

$$\langle N \rangle = 100/(2 \times RLOS) \quad (1)$$

where RLOS is the percentage of relative loss of signal found before and after Mn^{2+} addition. The principle of this method is comparable to the spin-label reduction method previously reported (12).

Cerebral Ischemia Model

Male Wistar rats (weighing 320-350 g), anesthetized by an i.p. injection of ethyl urethane (1.2 g/kg body weight), were made ischemic by bilateral clamping of the common carotid arteries for 20 min, after which the blood flow was definitively restored in all groups studied for survival rate. The animals were divided into groups of 40 animals. Two groups were treated with the liposomal formulation containing CDP_c, two groups with the free drug, and the other two groups with saline. Two groups were simply sham-operated (control groups). The liposome formulation, containing a dose of 20 mg/kg CDP_c, or the same dose of the free drug was administered by injection into the tail vein 1h before ischemia, immediately after and thereafter once a day for 6 days.

Rats investigated for brain lipid peroxidation were treated with CDP_c (alone or entrapped in liposomes) 1h before ischemia and then killed by decapitation after 20 min reperfusion. Brain lipid peroxidation was estimated by measuring the levels of conjugated diene, as previously reported (10 and references cited therein). The results were expressed as mmoles lipohydroperoxide/mg protein or mg lipid using ϵ_M 2.52 × 10^4 M^{-1} cm^{-1} (13).

Brain and Blood CDPc Distribution

Ischemized male Wistar rats (320-350 g) were treated after reperfusion by injection, via tail vein, with 20 mg/kg of labeled CDPc. ^{14}C-CDPc had a specific activity of 4 mCi/g. At different times (5 min, 30 min, 1h, 1.5h, and 2h) after the drug administration, the animals were killed. The brain and blood were immediately collected and weighed in glass vials. The biological tissues were homogenized in Soluen-350 (Packard) (1 ml/200 mg of biological material). The complete solubilization took 3 days, after which the radioactivity of the samples was measured in a scintillation counter. Part of the brain was employed to determine the radioactivity associated to the various phospholipid fractions.

The separation of the rat-brain lipids was carried out by chromatography. The cerebral tissue was homogenized with chloroform/methanol (2:1 v/v), 19 ml of solvent mixture/g of brain being used. The homogenate was filtered through a sintered-glass funnel (porosity 2), and the residue was washed with 5 ml of solvent/g of brain. The organic phase was evaporated off to dryness under reduced pressure, and the residue was dissolved in chloroform/methanol/water (64:32:4 v/v/v) and again evaporated to dryness. This procedure was repeated once more in order to decompose the proteolipids. Finally, the residue was suspended in chloroform/methanol (1:1 or 98:2 v/v). Proteins were removed by filtration through a sintered-glass funnel (porosity 3). The filtrate, diluted to 25 ml, was stored overnight at 2°C and again filtered to achieve a clear solution of brain lipids. The chromatography separation was carried out on silica acid or alumina column as reported elsewhere (14).

RESULTS AND DISCUSSION

Liposome Characterization

The lipid composition of the liposome system employed

throughout this work was DPPC/DPPS/CH (7:4:7 molar ratio). In fact, we previously reported (10) that this phospholipid mixture showed not only the best encapsulation efficiency parameters both as multilamellar vesicles and reverse-phase evaporation vesicles (EC = 5.7 and 15.5, respectively), but also the most suitable biological response in rat cerebral post-ischemic reperfusion. Ganglioside G_{M1} was introduced in the liposome lipid composition to confer a long-circulating property to SUVs (15-16). As in the case of anti-tumor chemotherapy (16), the presence of ganglioside G_{M1} may ensure a suitable penetration of the liposome formulation through the BBB, particularly if the vesicle suspension presents a mean size less than 100 nm. In fact, during an ischemic event there is damage to the BBB with the formation of fenestrations (about 100 nm in size), which leads to a hyperpermeabilization of the barrier and allows the permeation of micro-aggregates (17).

In order to achieve a liposome suspension presenting a mean size of about 50 nm with a narrow size distribution, the extrusion technique through polycarbonate filters was employed. This liposome preparation procedure was coupled with a freeze and thaw procedure to ensure high drug trapping efficiency (18). The EC value of SUVs was of 28.5. This value was higher than that expected (by about 5) for this type of liposome (19), owing to the ability of CDPc to form hydrogen bonds with the hydrophilic heads of charged phospholipids and particularly with DPPS (6-8).

The extrusion of MLVs through the polycarbonate filters ensured the formation of a highly monodispersed lipid vesicle suspension (polydispersity index = 0.01) with a mean size of 50 nm. ^{31}P-NMR assay showed a 50% relative loss of signal, thus showing the presence of unilamellar vesicles. As shown in Figure 1, the liposome extrusion through polycarbonate filters of different pore size (from 400 to 50 nm) caused a gradual reduction of both mean size and lamellarity.

The DPPC/DPPS/CH (7:4:7 molar ratio) SUVs presented a suitable phosphate buffer and serum stability. After 10 h a CDPc release plateau of 25% was reached (data not reported). The SUV serum stability was due to the inclusion in the lipid matrix of CH, ganglioside G_{M1} and solid-phase phospholipids such as DPPC and DPPS (16).

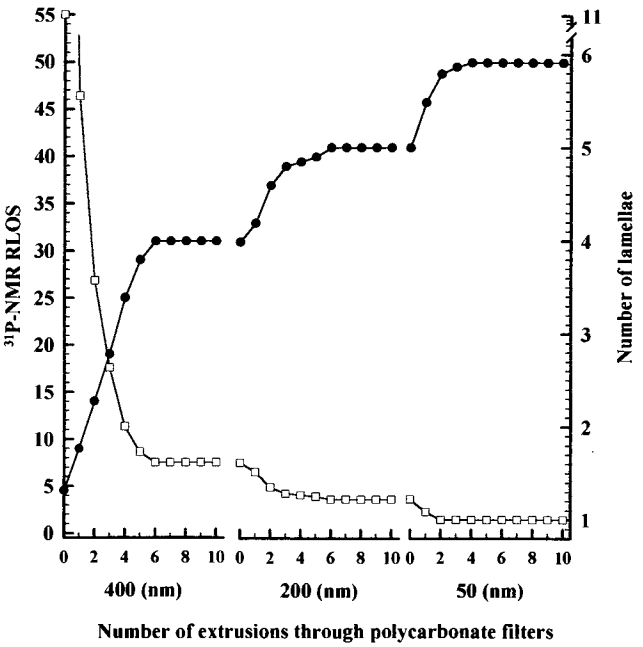

Fig. 1. Percentage of relative loss of signal (RLOS) after Mn^{2+} addition and lamellarity variation for MLVs passed through different polycarbonate filters of defined pore size (400, 200 and 50 nm) as a function of the number of passes. The phospholipid concentration was 50 mg · mL^{-1}. The extrusion was carried out at 50°C.

Therapeutic Effectiveness of CDPc-Loaded Liposomes

The *in vivo* experimental model, reported here, reproduces most of the aspects that normally occur in the two types of ischemia in humans: one associated with cardiac arrest and the other due to stroke (20). As reported in figure 2, the liposome formulation had a better biological effectiveness compared to the free CDPc, ensuring an improvement in the survival rate of ischemized and reperfused rats of approximately 66%. The data showed a discrepancy with the experiments previously reported (10), in which an improved survival rate of ~33% was achieved. However, in the latter study, one month old Wistar rats (80-100 g) were employed for the *in vivo* experiments. In these young animals the BBB is not completely formed, allowing a better permeation of both the free and liposome encapsulated drug. In the present study, vesicles with a size of 50 nm and a polydispersity index of 0.01 were used. The reduced size of SUVs compared to liposomes (120 ± 11 nm, polydispersity index = 0.1) previously reported (10), could ensure a better passage through the BBB fenestrations, which were formed as a consequence of the ischemic event. In fact, it is very improbable that particles with a mean size greater than 100 nm are able to pass the BBB even after ischemic injury (17).

In order to evaluate the biological effectiveness of re-

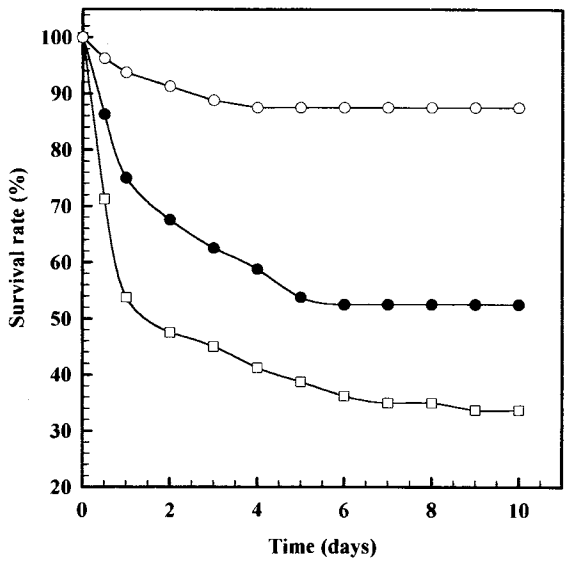

Fig. 2. Survival rate of Wistar rats (320-350 g) following post-ischemic reperfusion as a function of time. The various animal groups (a total of 80 animals for each experiment) were treated with free CDPc (●), drug-loaded SUVs (○) or simply with saline (□). Control group (sham-operated rats) had a survival rate of 100%.

ducing the lipid brain peroxidation, the conjugated diene levels in rat cerebral cortex after post-ischemic reperfusion were determined. This parameter is closely linked to the neuronal damage caused by the ischemic process; therefore, a reduction in the conjugated diene levels is an indicator of repair in biological membranes and of the activation of a functional reorganization (21). CDPc was able to lower the conjugated diene levels after post-ischemic reperfusion of rats. This effect was enhanced when the drug was incorporated into lipid vesicles. In fact, as reported in table I, the CDPc-loaded SUVs showed the lowest value of conjugated dienes. These findings demonstrated that the treatment with CDPc-loaded SUVs ensured a noticeable reduction of brain lipoperoxidation and decreased, as a consequence, the neuronal membrane damage caused by phospholipid degradation. Therefore, CDPc-loaded liposomes showed a greater protection against injury during reperfusion following ischemia than the free drug. The simple lipid vesicle suspension was also quite effective. Moreover, the suitable protection function exerted by the SUVs entrapping CDPc could be due to a synergic effect between the components of the lipid delivery devices and the drug. In fact, the therapeutic action of CDPc is mainly due to two factors: (i) biochemical activation of neuronal phospholipid synthesis and (ii) physiological increase of the cerebral blood flow (4). The latter ensured the scavenging of neurotoxic compounds formed during the ischemic event, such as excitatory amino acids, oxygen free radical species and Ca^{++}, which lead to neuronal degeneration and death. The CDPc biochemical action directly affects the damaged neurons allowing a rapid structural reorganization of the nervous system. In this case, the neuron cells can employ the substrate available in the environment, as phospholipids of the liposome formulation, to repair and reactivate the neuronal membranes.

Blood and Cerebral CDPc Distribution

The improvement of the therapeutic effectiveness, namely the number of rats which survive ischemia, and the cerebral cortex conjugated diene levels of CDPc-loaded SUVs compared with the free drug was due to the enhanced delivery of the biologically active compound to the target site (cerebral districts), as shown in Figure 3. The liposome formulation ensured that 22% of the injected dose entered the

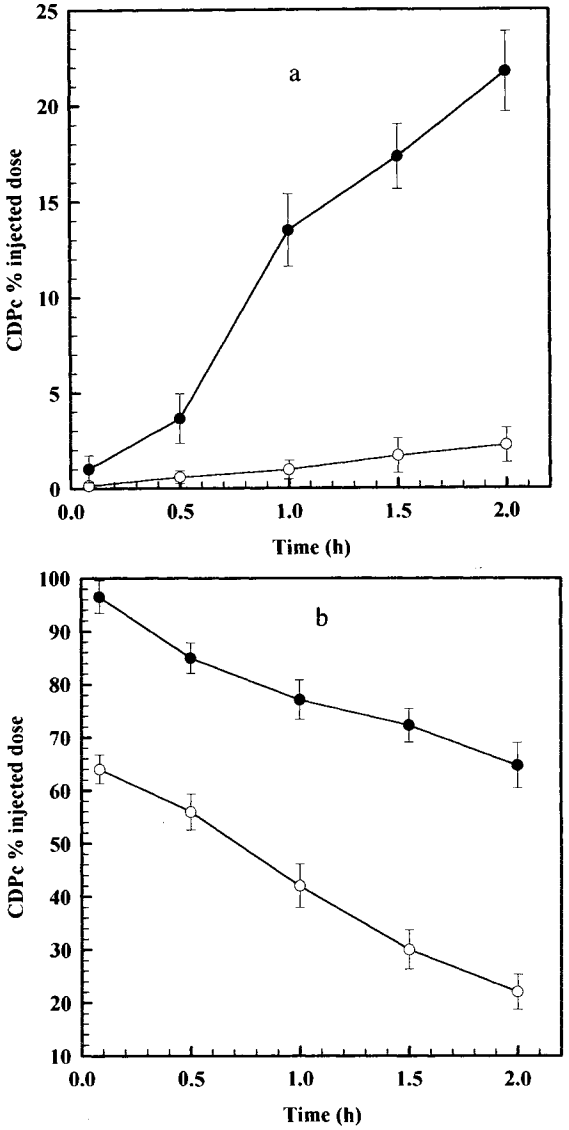

Fig. 3. Brain (a) and blood (b) distribution of free (○) and liposome encapsulated CDPc (●) as a function of time in post-ischemic reperfused rats. The administration was carried out via tail vein. Each value represents the average ± S.D. of six experiments (three male and three female).

Table I. Conjugated Diene Levels as an Index of Brain Lipoperoxidation in Rat Cerebral Cortex After Post-Ischemic Reperfusion

Experiment[a]	Lipohydroperoxide in proteins (mmol · mg protein^{-1})	Lipohydroperoxide in lipids (mmol · mg lipid^{-1})
Control (Sham-operated)	18.1 ± 2.1	53.2 ± 4.1
Saline treated rats	40.7 ± 6.3[b]	109.6 ± 10.4[b]
Free CDPc treated rats	25 ± 3.2[c]	61.3 ± 3.2[c]
Liposome treated rats		
without CDPc	13.4 ± 1.4[c]	27.6 ± 1.4[c]
with CDPc	2.9 ± 0.8[c,d]	3.7 ± 1.1[c,d]

[a] Each value is the average of six animals ± S.D.
[b] $P < 0.001$ compared with control.
[c] $P < 0.001$ compared with saline treated rats.
[d] $P < 0.001$ compared with rats treated with free CDPc.

cerebral districts after the ischemic event. This value is 11 times greater than that of free CDPc (only 2% of the injected dose).

Table II summarizes the level of choline incorporated into the various cerebral lipid fractions 2 h after the administration of free or liposome entrapped drug in post-ischemic reperfused rats. The percentage distribution of the drug in the lipidic components of the brain remained almost constant, showing that the liposomal drug delivery device did not influence the distribution patterns of CDPc in the various cerebral lipid fractions.

CONCLUSIONS

The survival rate and the conjugated diene levels in rat cerebral cortex after post-ischemic reperfusion showed that CDPc-loaded liposomes can not only enhance the number of rats surviving after an ischemic event, but also allow an almost complete recovery of the neuronal functionality, particularly of the neurons present in the border zone, directly damaged by the oxygen free radicals and high levels of excitatory amino acids. This is of particular importance in humans, where, besides survival, a serious problem is the quality of life after an ischemic event. In fact, invalidism is the result of the degradation of an area of the cerebral cortex triggered by the ischemic event. This area is constituted by the ischemic focus (zone damaged by the blockage of oxygen and glucose -ischemia-) and of the penumbra zone (area damaged by neurotoxic compounds generated by ischemia). Therefore, the remarkable reduction of conjugated diene levels is a guarantee of neuron survival ensuring a rapid and almost complete reorganization of the nervous structures. In our opinion, the best therapeutic response could be ascribed to two factors: (i) the enhanced delivery of CDPc to the cerebral districts, and (ii) the possibility for the liposomal formulation to act as a circulating reservoir which may slowly favor the drug entrance into the brain. In fact, the characteristic of prolonged circulation time in the blood of the SUV formulation may ensure a prolonged supply of the therapeutic substrates (CDPc and phospholipids) by means of repeated passages through the cerebral districts. Considering the encouraging results, a clinical application is envisaged.

Table II. Incorporation of Free or Liposome Encapsulated ^{14}C-CDPc in the Various Fractions of Cerebral Lipids 2 h After the Administration of the Radiolabeled Drug[a]

Cerebral lipid fraction	Free CDPc (%)[b]	CDPc-loaded liposome (%)[b]
Chephalines	27.3 ± 4.3	20.6 ± 3.1
Lecithines	69.4 ± 9.2	75.8 ± 10.3
Sphingomyelines	3.3 ± 1.7	3.6 ± 1.9

[a] Each value is the average of six experiments (three male and three female) ± S.D.

[b] The amount of ^{14}C-CDPc incorporated in the various cerebral lipid fractions is expressed as the percentage of total amount incorporated in the cerebral tissues.

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