Pharmacology and Toxicity of Intracarotid Adriamycin Administration Following Osmotic Blood-Brain Barrier Modification

Edward A. Neuwelt, Michael Pagel, Peggy Barnett, Mark Glassberg, and Eugene P. Frenkel

ABSTRACT

The effect of reversible blood-brain barrier modification on the delivery of Adriamycin to the brain was studied in a rodent and canine model. Pharmacokinetic and physiological studies were done in these animals after a wide range of doses of Adriamycin (0.1 to 1.0 mg/kg) were administered into the carotid artery following osmotic barrier modification with mannitol. In the absence of barrier modification, no immunoreactive Adriamycin was detected in the cerebrum; whereas, following barrier modification, up to 4.5 μg of drug and/or metabolites per g of brain were found. Optimum tissue levels of Adriamycin and metabolites were achieved following barrier modification when the drug was administered by either bolus or slow continuous (15-min) infusion. Immunoreactive drug was identified in brain for up to 6 hr after administration. Significant functional neurotoxicity occurred at all dose levels, even at 0.1 mg/kg, a level at which Adriamycin concentration in the brain was below the level of detectability. Neuropathological examination revealed the presence of necrosis and hemorrhagic infarcts. Thus, these pharmacological and toxicity studies suggest that Adriamycin (or its metabolites) may produce significant clinical neurotoxicity when even small amounts penetrate the blood-brain barrier.

INTRODUCTION

Adriamycin, like most other chemotherapeutic agents, penetrates the normal blood-brain barrier poorly (8). Even in tumors in the brain where the blood-brain barrier is usually at least partially altered, systemic drug administration appears to result in inadequate drug delivery both to the tumor and the immediate surrounding brain (2). This is clinically exemplified in a report by Benjamin et al. (1) of a series of patients in whom Adriamycin failed to control metastasis to the brain from sarcomas while that drug resulted in evidence of responses to the tumor in nonbrain sites.

One possible approach to improving this drug delivery to brain is to bypass the blood-brain barrier by administering the drug directly into the cerebrospinal fluid. This approach has 2 problems. The first is the fact that drug given intraventricularly or intrathecally only attains therapeutic levels in the outer few mm of the cortex because the diffusion into the parenchyma is slowed by the small extracellular space (2, 6, 9, 20, 22, 23). The second problem is that in order to attain sufficient diffusion gradients, the doses administered must be at very high, or essentially toxic, levels. Merker et al. (8) infused Adriamycin into the cerebrospinal fluid spaces of the Rhesus monkey. At inflow concentrations of 6 to 100 μg/ml, death or severe toxicity was seen by the tenth postinfusion day, and a distinctive noninflammatory necrotizing angiopathy was seen.

Previous animal and clinical studies from this laboratory have demonstrated the value of hyperosmolar mannitol in producing transient blood-brain barrier modification as a means of increasing the drug concentration of methotrexate or iodinated contrast agents in the brain (10-16). The purpose of the present study was to evaluate the effect of blood-brain barrier modification on Adriamycin delivery to brain.

MATERIALS AND METHODS

Canine Model

Blood-Brain Barrier Modification. Adult mongrel dogs (20 to 25 kg) were used in the acute studies and conditioned hound dogs (20 to 25 kg) in the chronic studies (Brink Kennels, Paola, Kans.). The dogs were anesthetized with sodium thiopental (20 mg/kg), intubated with an endotracheal tube, and ventilated with a Harvard animal respirator (Harvard Apparatus Co., Inc., Millis, Mass.). Anesthesia was maintained with a 60% nitrous oxide:oxygen mixture and supplemental sodium thiopental. An i.v. catheter (18-gauge) was used for anesthetic drug infusion and fluid management. Intraoperatively, atropine sulfate (0.015 mg/kg, i.v.) and Lasix (5 mg, i.v.) (furosemide; Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N. J.) were administered.

Blood-brain barrier modification was performed using the technique described previously by this laboratory (14-16). The left internal carotid artery was canulated with a 16-gauge catheter via the common carotid artery. Fifteen min before barrier disruption, Evans blue (2%; 3 ml/kg) was administered i.v. Evans blue was used as a marker dye because it is known to bind tightly, but reversibly, to plasma albumin, and it therefore does not normally penetrate the tight junctions between cerebral endothelial cells (18). Mannitol (25%) (Merck Sharp & Dohme, West Point, Pa.) at 37° was filtered (0.45-μm pore diameter; Nalco Co., Rochester, N. Y.) and then infused into the internal carotid artery at a rate of 1.5 ml/sec over 30 sec. In control animals, a 0.9% NaCl solution instead of the mannitol was infused at an identical rate and volume. Five min after either of the above, Adriamycin (doxorubicin HCl; Adria Laboratories, Inc., Columbus, Ohio) was infused over a 15-min period. In the dose-ranging studies, the Adriamycin dose extended from 0.1 to 1.0 mg/kg.

Acute animal studies are defined as those animals killed 1 hr after the intracarotid infusion of either 0.9% NaCl solution or mannitol. At sacrifice, the brain was removed and sliced to evaluate the distribution of Evans blue staining, and samples of contralateral and ipsilateral gray matter, white matter, and basal ganglia were obtained to measure Adriamycin concentration.

Chronic animal studies are defined as those conditioned animals permitted to awaken after the procedure, serially examined to evaluate...
toxicity, and then studied for neuropathological sequelae. These animals were observed for up to 33 days prior to sacrifice. At sacrifice, the brain was removed, evaluated for Evans blue staining, and then fixed in Caron's formalin for histopathological examination.

Rodent Model

Blood-Brain Barrier Modification. Adult rats (Osborn-Mendel strain), weighing 250 to 300 g, were used for the study of normal animals. Blood-brain barrier modification was performed using the technique of Rapoport (17, 19) with minor modifications. Animals were anesthetized with sodium pentobarbital (40 to 50 mg/kg, i.p.). A catheter filled with sodium heparin in isotonic 0.9% NaCl solution was tied into the right external carotid artery for retrograde infusion. Five min before blood-brain barrier disruption, Evans blue (chroma-Gesellschaft, Stuttgart, West Germany) was administered i.v. (2%; 2 ml/kg). Mannitol (25%).(Merck Sharp & Dohme), warmed to 37°, was infused for 30 sec cephalad into the right internal carotid artery via the external carotid artery catheter at a rate of 0.12 ml/sec (19). In control studies, 0.9% NaCl solution instead of mannitol was infused at an identical rate and volume (0.12 ml/sec for 30 sec).

Determination of the Optimal Timing of Adriamycin Administration Relative to Barrier Modification. Adriamycin (1 mg/kg) was administered to Osborn-Mendel rats intraarterially by 2 different methods to evaluate its brain permeability characteristics. One group received a bolus injection of Adriamycin (diluted in 1-ml volume) over 30 sec at time intervals of either 5 sec, 5 min, 10 min, 30 min, or 45 min after intracarotid infusion of either 0.9% NaCl solution or mannitol. The second group had a constant 15-min Adriamycin infusion (diluted to 3-ml volume) which was begun 5 min after mannitol or 0.9% NaCl solution. All animals were sacrificed 1 hr after intracarotid 0.9% NaCl solution or mannitol infusion. Serum and tissue samples from each hemisphere were collected, and the extent of Evans blue staining was evaluated.

Time Course of Disappearance of Adriamycin from Brain. Adriamycin (1 mg/kg) was infused into Osborn-Mendel rats for 15 min through the external carotid artery catheter 5 min after intracarotid infusion of mannitol or 0.9% NaCl solution. Animals were subsequently sacrificed at 30 min, 1 hr, 3 hr, and 6 hr; sections of brain were removed for evaluation of Evans blue staining, and serum and brain tissue were aliquoted for determination of drug content.

Evaluation of the Degree of Blood-Brain Barrier Modification. In all animals, the degree of staining of each hemisphere after the administration of Evans blue was graded as follows: Grade 0, no staining; Grade 1+, just noticeable staining; Grade 2+, moderate blue staining; and Grade 3+, dark blue staining. The staining has been shown previously to correlate with the degree of the "blood-brain barrier" modification (i.e., drug delivery) (14-17).

Adriamycin Radioimmunoassay

The high lipid content of brain tissue and the lipophilic character of Adriamycin posed problems in direct application of the radioassay method. The extraction was essentially as described from this laboratory for other types of cells (21). An initial extraction was performed in 10 volumes of chloroform:methanol (4:1). The organic phase was removed and evaporated on a Labconco desiccator (Labconco Corp., Kansas City, Mo.) at room temperature, and the residue was saved for assay. The radioimmunoassay was performed by kit method (Diagnostic Biochemistry, San Diego, Calif.) on the tissue extracts obtained as described above. The extracts were reconstituted in a 0.9% NaCl solution:gelatin buffer. An internal standard was prepared by adding Adriamycin to a tissue extract, and the aliquots of standard were frozen at -80° until used.

The assay procedure consists of 50 Rl of standard or unknown sample, 50 Rl of an 125I-labeled Adriamycin derivative (5000 cpm), and 200 Rl of a dilution of a goat Adriamycin antibody in 12- x 75-mm borosilicate glass tubes. The assay mixtures were incubated 1 hr at 4°. Bound and free drug were separated by the addition of 250 Rl dextran-coated charcoal (1% Norit A and 0.025% Dextran T-80) at 0-4°. Five min after the addition of charcoal, the tubes were centrifuged at 3000 rpm (1500 x g). The supernatant fluid was decanted into 13- x 100-mm tubes and subsequently assayed for 125I in a Beckman 4000 spectrometer. All samples were done in duplicate and were counted to at least 10,000 total counts.

Data were analyzed after logit transformation,

\[
\ln \left( \frac{B/Bo}{1 - B/Bo} \right)
\]

where Bo is the percentage bound in the absence of standard Adriamycin and B is the sample or standard binding. Both B and Bo were corrected for nonspecific binding (complete assay minus antibody). Nonspecific binding with the assay ranged between 2.6 and 8.2% (average, 6.2%). Bo (corrected for nonspecific binding) ranged between 42.6 and 67.8% (average, 54.5).

Assay aliquot volumes were maintained at or below 5-μl equivalent of brain tissue (50-μl equivalent of chloroform:methanol extract), which is a 1.25-mg aliquot of brain tissue. At this level, nonspecific binding was minimal. The limit of resolution of the assay at these volumes was 0.02 μg of Adriamycin per g of brain tissue.

To test the adequacy of our extraction, tracer amounts of [3H]-daunorubicin (1.0 Ci/mmol; New England Nuclear, Boston, Mass.) or [3H]Adriamycin (1.0 Ci/mmol; generously provided by S. Gupta of New England Nuclear) were added to tissue homogenates and subjected to the extraction procedure, and the recovery was quantitated by [3H] assay or by radioimmunoassay of the [3H]-labeled compound. Aquasol (New England Nuclear) containing 10% methanol provided complete dissolution of [3H]anthracycline for scintillation counting. When known amounts of [3H]Adriamycin were added to tissue homogenates and subjected to the chloroform:methanol extraction, an average recovery of 94.2 ± 12.9% (S.D.) in 9 separate experiments was obtained. In addition, the assay of known amounts of radioinert Adriamycin (10 ng/ml) from a tissue homogenate containing 1.25 mg of brain and subjected to chloroform:methanol (4:1) extraction yielded an average value of 9.5 ng/ml in 5 separate studies.

In an evaluation of 15 specimens, the intraassay coefficient of variation was 7.0% and the interassay coefficient was 11.3%. The cross-reactivity of the antibody is such that related metabolites of Adriamycin (adriamycinol, Adriamycin aglycone, and other metabolites) are also measured by this assay.

RESULTS

Evaluation of Optimal Delivery of Adriamycin to Rodent Brain. The initial studies of Adriamycin focused upon the optimal temporal relationships for the drug administration following mannitol infusion. In addition, bolus administration was compared to prolonged infusion administration.

As shown in Chart 1, immunoreactive Adriamycin brain levels (i.e., Adriamycin plus metabolites) in the rodent were significantly higher following osmotic blood-brain barrier modification than those achieved in 0.9% NaCl solution control animals with an intact blood-brain barrier where drug levels were virtually unmeasurable. In previous studies with other agents such as methotrexate (14), the standard infusion technique was to infuse the test drug over a 15-min period beginning 5 min after intracarotid 0.9% NaCl solution or mannitol infusion. When Adriamycin was given in this manner at a dose of 1.0 mg/kg, the brain Adriamycin concentration ranged between 0.2 and 0.35 μg/g of tissue. This method of administration was then compared to "bolus administration" of the same amount of drug given immediately after the mannitol infusion and infused over a very brief period (30 sec). As shown in Chart 1, bolus infusion resulted in brain Adriamycin levels between 0.26 and
Chart 1. Brain Adriamycin levels utilizing 2 different methods of intraarterial administration in the rat following blood-brain barrier disruption. ——, mean brain values for rats given Adriamycin (1 mg/kg) for 30 sec at the given time point after blood-brain barrier disruption; bars, S.E.; [3] ± S.E. for rats given intracarotid Adriamycin (1 mg/kg) for 15 min starting 5 min after blood-brain barrier disruption. Both rapid and slow infusion groups of animals were sacrificed 1 hr after blood-brain barrier disruption with mannitol. In control animals (not shown) perfused with 0.9% NaCl solution and sacrificed 1 hr later, no detectable levels of immunoreactive Adriamycin were observed regardless of whether Adriamycin was given by rapid intraarterial bolus or slow infusion.

0.47 µg of immunoreactive Adriamycin per g of tissue. Of considerable interest is that the slight advantage of rapid bolus infusion, over the 15-min infusion, was lost within a few min of the mannitol barrier modification. That is, giving the Adriamycin as a 30-sec bolus but beginning the infusion 10 min after the mannitol resulted in dramatically less drug delivery than when either of the 2 approaches described above was used. Since rapid bolus infusion did not confer a great delivery advantage over the standard 15-min infusion method, the latter was chosen for logistic ease and because it was the mode used in our previous studies, thereby permitting comparative analyses.

Pharmacokinetics of Adriamycin Delivery after Osmotic Blood-Brain Barrier Disruption in the Rodent. The fate of immunoreactive Adriamycin in the brain following delivery (1.0 mg/kg) with the barrier modification technique was studied. Serial samples obtained over the first 6 hr following injection demonstrated (see Chart 2) the rapid decline of immunologically reactive Adriamycin in the brain in those animals given the drug after mannitol barrier modification. No drug was identifiable at any time point in the brain of control animals given the Adriamycin after 0.9% NaCl solution infusion. In the mannitol animals, the mean Adriamycin concentration at 30 min was 0.44 µg of Adriamycin per g of brain, and by 6 hr postinfusion Adriamycin was essentially undetectable (Chart 2). In those animals infused with mannitol, the Adriamycin concentration in the contralateral cerebral hemisphere was at the limits of sensitivity of our assay. The peak concentrations ranged from 0.01 to 0.025 µg of drug per g of tissue.

Effect of Adriamycin in the Canine When Administered into the Internal Carotid Artery Following Osmotic Blood-Brain Barrier Disruption. To evaluate possible Adriamycin neurotoxicity in the canine, Adriamycin was administered in dose-ranging studies extending from 0.1 to 1.0 mg/kg (Table 1). Drug was given into the internal carotid artery 5 min after the mannitol infusion and was infused over a 15-min period. All animals given 1.0 mg/kg after barrier modification developed seizures (Table 1). In 3 of 4 animals, seizures developed at 7 to 8 days following drug administration. In one animal, the seizures developed 24 days following drug administration. There was evidence of Evans blue-albumin staining at the time of sacrifice in all of these animals, indicating that osmotic blood-brain barrier disruption had been successful. At necropsy examination, hemorrhage and necrosis of ipsilateral brain were seen in all the animals. For comparison, a control (0.9% NaCl solution-infused) animal that was given Adriamycin (1 mg/kg) was observed for 33 days with no neurological sequelae, and at postmortem examination neither Evans blue staining nor histological changes were seen.

At Adriamycin levels of 0.5 mg/kg, 3 of 5 animals developed neurological toxicity. Two animals did not regain consciousness, and histological examination showed severe hemorrhagic necrosis of brain. In the third animal, seizures developed 7 days following drug administration, and examination revealed cerebral necrosis. Two animals were studied at 0.25 mg/kg and 2 at 0.1 mg/kg. One of the 2 animals in each group developed similar neurological and histopathological changes.

As shown in Table 2, the duration of these studies (2 hr to 33 days) only permitted a retrospective evaluation of the adequacy of the osmotic blood-brain barrier disruption using the degree of Evans blue-albumin staining. Because of the variable time to sacrifice, staining was graded as either present (+) or absent (0). In addition (Table 1), 3 animals had only barrier modification without subsequent Adriamycin; no neurological or pathological sequelae from the barrier modification procedure were seen.

Brain Adriamycin Levels in the Canine after Varying Doses of Intracarotid Adriamycin. Since the observed toxicity could have been due to inordinate brain levels of Adriamycin or to exquisite brain sensitivity to the drug, the concentration of Adriamycin in brain was compared to the levels found at extraneural sites in dogs and humans.

The concentration of Adriamycin and its metabolites found in the brain and serum are summarized in Table 2. The Adriamycin levels for each drug dose were highest in the gray matter, intermediate in the white matter, and lowest in the basal
Table 1

<table>
<thead>
<tr>
<th>Adriamycin dose (mg/kg)</th>
<th>Neurological deficits</th>
<th>Pathological findings</th>
<th>Time of sacrifice after blood-brain barrier disruption</th>
<th>Blood-brain barrier disruption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (mannitol infused)</td>
<td>0</td>
<td>None</td>
<td>None</td>
<td>30 days +</td>
</tr>
<tr>
<td>Control (0.9% NaCl solution infused)</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>30 days +</td>
</tr>
<tr>
<td>Experimental (mannitol infused)</td>
<td>1</td>
<td>Seizures at 8 days</td>
<td>Microscopic areas of necrosis</td>
<td>28 days +</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Seizures at 7 days</td>
<td>Microscopic areas of necrosis</td>
<td>13 days +</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>None</td>
<td>None</td>
<td>33 days +</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Never regained consciousness</td>
<td>Bilateral hemorrhagic necrosis; left parietal cystic cavity</td>
<td>8 days +</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>None</td>
<td>None</td>
<td>31 days +</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Never regained consciousness</td>
<td>Hemorrhagic infaracts</td>
<td>24 hr +</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Circled to left, seizures at 7 days</td>
<td>Multiple areas of necrosis</td>
<td>7 days +</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>None</td>
<td>None</td>
<td>7 days +</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>None</td>
<td>None</td>
<td>31 days +</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>Never regained consciousness</td>
<td>Multiple areas of hemorrhage</td>
<td>2.5 hr +</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>None</td>
<td>None</td>
<td>33 days +</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>Never regained consciousness</td>
<td>Multiple areas of hemorrhage</td>
<td>2.5 hr +</td>
</tr>
</tbody>
</table>

* The success of blood-brain barrier disruption was determined by the presence or absence of Evans blue staining at sacrifice.

Table 2

<table>
<thead>
<tr>
<th>Adriamycin concentration in canine brain 1 hr after blood-brain barrier modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin concentration* (µg/g)</td>
</tr>
<tr>
<td>Contralateral hemisphere</td>
</tr>
<tr>
<td>Adriamycin dose (mg/kg)</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>1 1+</td>
</tr>
<tr>
<td>0.5 1+</td>
</tr>
<tr>
<td>0.5 2+</td>
</tr>
<tr>
<td>0.5 1+</td>
</tr>
<tr>
<td>0.5 2+</td>
</tr>
<tr>
<td>0.5 3+</td>
</tr>
<tr>
<td>0.5 3+</td>
</tr>
<tr>
<td>0.25 2+</td>
</tr>
<tr>
<td>0.1 3+</td>
</tr>
</tbody>
</table>

* Adriamycin was administered over 15 min beginning 5 min after mannitol infusion.
  * 0, no staining; 1+, just noticeable staining; 2+, moderate staining; 3+, dark blue staining.
  * ND, below analytical limits.

The degree of osmotic blood-brain barrier disruption, the greater is the amount of drug delivered, further emphasized by the very low drug concentration in brain in those animals at 0.5 mg/kg in which there was poor barrier disruption (Table 2).

The serum levels in the dogs receiving 0.5 to 1 mg of Adriamycin per kg ranged from 0.5 to 6.9 µg/ml. Liver Adriamycin levels were measured in the animals receiving 0.5 mg/kg, and hepatic concentration ranged from 0.5 to 3.3 µg/g of liver.
Despite good blood-brain barrier disruption, no detectable Adriamycin was seen in the brain in 2 animals that were given Adriamycin infusions of 0.1 to 0.25 mg/kg. These animals had serum Adriamycin levels of 0.26 and 0.35 μg and hepatic concentration of 0.125 μg Adriamycin per g of liver.

**DISCUSSION**

The efficacy of Adriamycin in the management of a variety of systemic tumors led to the present studies. It is clear that the delivery of Adriamycin to the cerebrum can be enhanced markedly in normal animals (rodents and dogs) when osmotic blood-brain barrier modification is used prior to drug administration. Even at high systemic concentrations of Adriamycin given into the carotid artery of 0.9% NaCl solution-infused control animals, little drug reached the brain. By contrast, with preparation by mannitol to achieve reversible transient blood-brain barrier modification, the Adriamycin levels in the brain were similar to those observed in the liver. Of particular note is that these hepatic concentrations are comparable to those described in patients on systemic Adriamycin therapy programs where therapeutic responsiveness of the tumor has been identified (7). In these studies, as in previous clinical reports (7), the rapid and variable serum clearance of Adriamycin appears to be the reason for the widely varying serum Adriamycin levels observed after 1 hr (i.e., Table 2).

To attain drug concentrations in the brain that begin to mirror those achieved in other parenchymal organs clearly requires a delivery mechanism that overcomes the problem of the blood-brain barrier (11) which may be partially or totally intact even in tumor (12). From the present studies, it is also clear that the temporal relationship of the drug delivery and its mode relative to the timing of barrier disruption is important. For Adriamycin, drug delivery needs to be performed either by rapid bolus immediately following barrier modification or by slow (15-min) infusion following barrier manipulation. The importance of this temporal relationship and mode of delivery is emphasized by the observation that bolus delivery of Adriamycin is associated with considerably less drug delivery when given more than 5 min after barrier modification. Recent observations from this laboratory have demonstrated that these factors (time following barrier modification and the mode of drug delivery) vary with moieties of different molecular size or shape (10, 14).

Immunoreactive Adriamycin and its metabolites were identifiable in the brain (Chart 2) parenchyma for up to 6 hr following administration by successful delivery techniques. The subsequent fate of the Adriamycin in the brain, be it intercalated into the DNA, metabolized, or lost from the cells, is not resolvable at this time.

Unfortunately, despite the success that osmotic blood-brain barrier disruption confers on delivery of Adriamycin, the current studies clearly indicate that Adriamycin is highly neurotoxic. Even at very low tissue levels, unacceptable functional and histological neurotoxicity were seen. These results are similar to those of Merker et al. (8), who described neurotoxicity in Rhesus monkeys when Adriamycin was delivered to the cerebrospinal fluid by ventriculomacular perfusion. That this is not due to routes of delivery is suggested by Cho et al., who demonstrated necrosis of dorsal ganglion cells (3, 5) and peripheral neuropathy (4) following conventional doses of i.v. Adriamycin in the rat. It is possible that the neurotoxicity seen with Adriamycin (or its metabolites) may not apply to other related anthracyclines.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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