



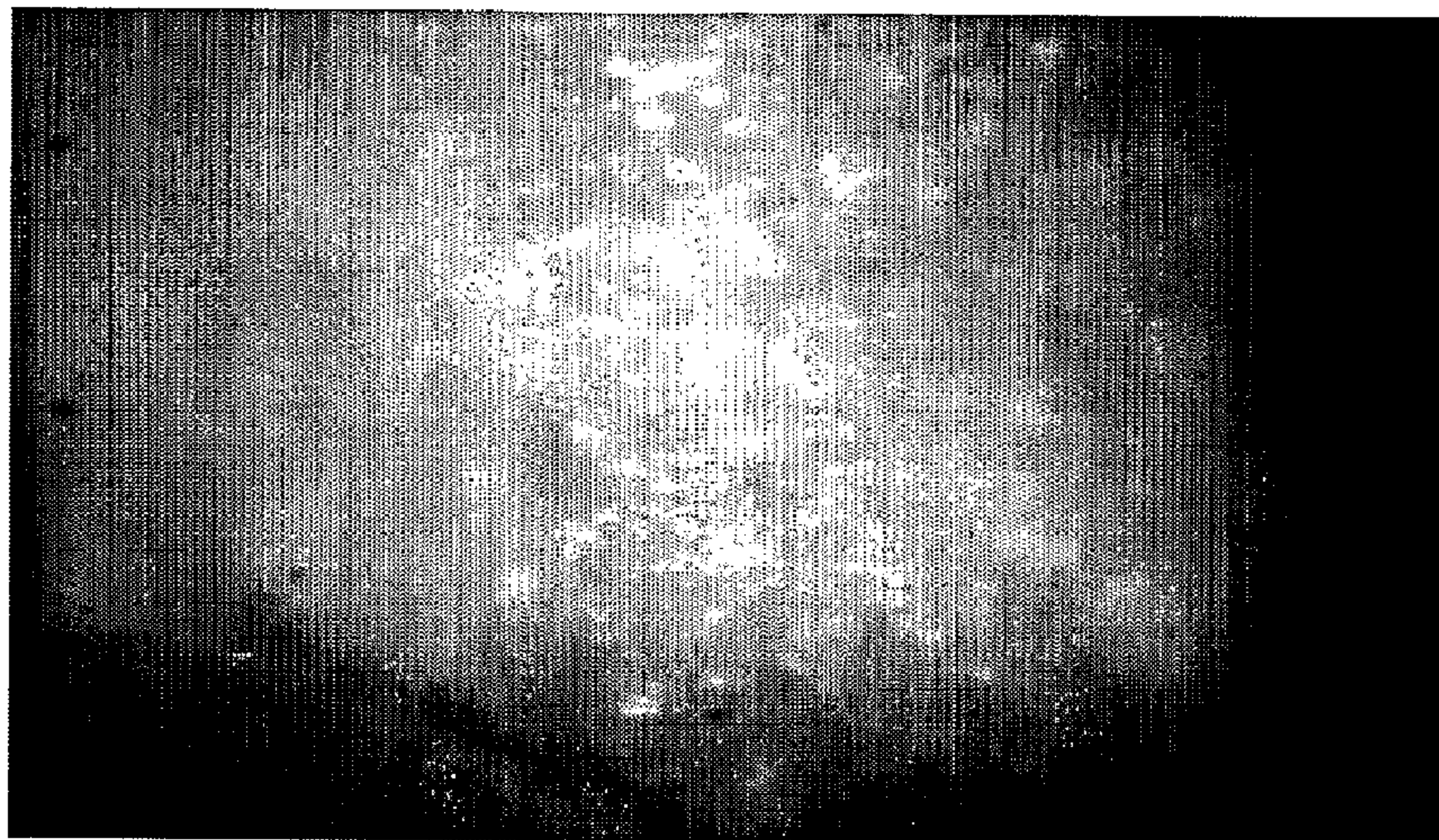
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(54) Titre : DETECTION D'APOPTOSE IN VIVO
(54) Title: IN VIVO DETECTION OF APOPTOSIS

SCK mammary tumor in A/J mice, injected intravenously with
8.0 µg of FAM-VAD-FMK, 3 Hours

Post Treatment Time: 3 Hours, ATO 8.0 mg/kg



(57) Abrégé/Abstract:

The invention provides methods and products, such as kits, useful for determining the apoptotic state of cells in an organism, comprising detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the presence or abundance of the caspase affinity labeling agent correlates with the apoptotic state of the cells.



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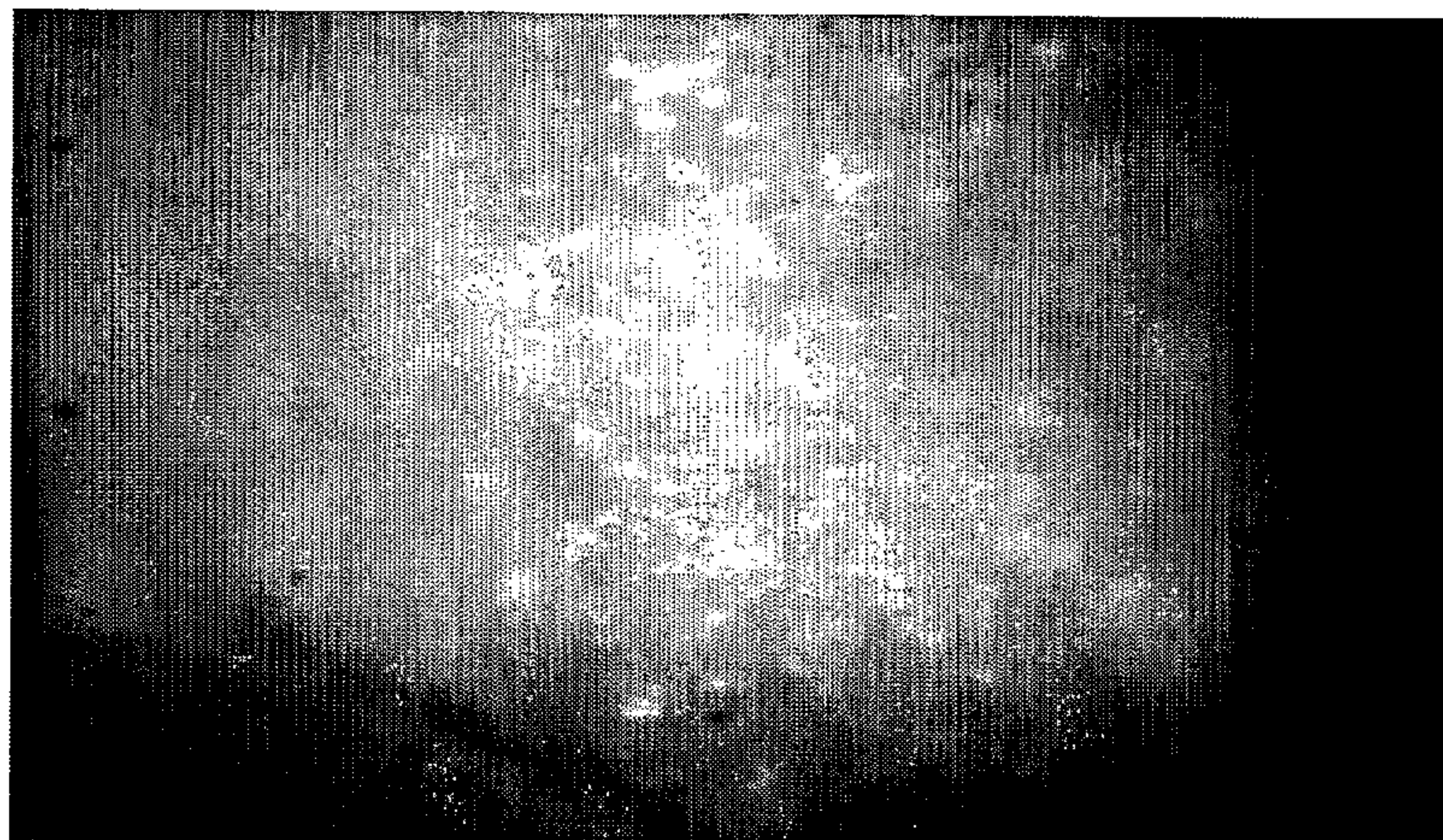
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Post Treatment Time: 3 Hours, ATO 8.0 mg/kg



(57) Abstract: The invention provides methods and products, such as kits, useful for determining the apoptotic state of cells in an organism, comprising detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the presence or abundance of the caspase affinity labeling agent correlates with the apoptotic state of the cells.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

IN VIVO DETECTION OF APOPTOSIS

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Related Application(s)

This patent document claims the benefit of priority of U.S. application serial No. 60/729,227, filed October 21, 2005, which application is herein incorporated by reference.

Background

10

There have been attempts at *in vivo* apoptosis detection and imaging using Annexin V and Annexin V derivatives (*see, e.g.,* Kietselaer *et al.*, 2003; Belhocine *et al.*, 2004; Reddy, 2005; Boersma *et al.*, 2005; Watanabe *et al.*, 2006; Vanderheyden *et al.*, 2006; and Corsten *et al.*, 2006). Other attempts using novel compounds that rely on the perturbation and alterations of the normal organization of the cell plasma membrane have also been attempted (*see, e.g.,* U.S. Patent Application Publications 2005/0244812 and 2005/0276750).

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None of the previously described methods uses a cell permeant probe for the detection and imaging of apoptosis. As a result of this and other issues, all of the aforementioned methods have been plagued with problems resulting in high backgrounds and lack of binding to certain apoptotic tumor cells. Annexin V is not cell permeant, is slow to penetrate any tissues, has high background, and does not detect early apoptotic cells (Kietselaer *et al.*, 2003; Belhocine *et al.*, 2004; Boersma *et al.*, 2005; Watanabe *et al.*, 2006; Vanderheyden *et al.*, 2006; and Corsten *et al.*, 2006). Leading to high background, Annexin V binds positively to normal and healthy bone marrow derived cells (Dillon, 2001). It has been reported that Annexin V does not bind to all tumor cells (Dicker, 2005).

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25

In the Ziv publications (U.S. Patent Application Publications 2005/0244812 and 2005/0276750), it is reported that their compounds accumulate in apoptotic cells at a rate faster than they accumulate in cells that are not undergoing cell wall turnover. This leads to high background levels and lack of specificity similar to Annexin V. Requiring a compromised cell state also prohibits the detection of cells that are in the early stages of apoptosis.

30

Thus, previously described methods of *in vivo* apoptosis detection and imaging lack specificity and sensitivity and are subject to high background. The use of sensitive and specific cell permeant inhibitor probes that bind to specific active enzymes and proteases involved in apoptosis has not been described.

5 Thus, methods and products for *in vivo* determination of the apoptotic state of cells in an organism are needed.

Brief Description of the Figures

Figure 1. Low magnification bright-field image composite of SCK mammary tumors taken through a window chamber. Each mouse was injected
10 with 2×10^5 SCK tumor cells. The tumors were allowed to grow for 7 days. A low level of hemorrhaging was seen in both control and test mice.

Figure 2. Low magnification bright-field image composite of SCK mammary tumors taken through a window chamber. The control mouse was injected with a placebo and the test mouse was injected with 8.0 mg/kg of
15 arsenic trioxide (ATO). Increased levels of hemorrhaging can be seen in the mouse that received the ATO treatment. The photographs were taken 3 hours after completion of treatment.

Figure 3. Low magnification bright-field image composite of SCK mammary tumors taken through a window chamber. The control mouse was
20 injected with a placebo and the test mouse was injected with 8.0 mg/kg of ATO. Increased levels of hemorrhaging can be seen in mice that received the ATO treatment, the amount of hemorrhaging has also increased over time when compared to the 3 hour post treatment photograph. The photographs were taken 24 hours after completion of treatment.

25 Figure 4. High magnification photograph using an excitation at 488 nm with a fluorescein filter to detect fluorescence. The mouse was injected with 2×10^5 SCK tumor cells. The tumor was allowed to grow for 7 days. The mouse was then injected intravenously (I.V.) through the tail vein with 8 μ g of the apoptosis detection reagent FAM-VAD-FMK prior to therapeutic treatment. The
30 reagent was allowed to circulate in the mouse for 30 minutes before photographing with 488 nm excitation. The results demonstrate a low level of apoptosis which is expected in a fast growing SCK tumor.

Figure 5. High magnification photograph using an excitation at 488 nm with a fluorescein filter to detect fluorescence. The mouse was injected with 8.0 mg/kg of ATO. After 3 hours post ATO treatment, the mouse was injected intravenously through the tail vein with 8 μ g of FAM-VAD-FMK. The reagent
5 was allowed to circulate in the mouse for 30 minutes before photographing with 488 nm excitation. The results demonstrate a high level of apoptosis.

Figure 6. High magnification photograph using an excitation at 488 nm with a fluorescein filter to detect fluorescence. The mouse was injected with 8.0 mg/kg of ATO. After 24 hours post ATO treatment, the mouse was injected
10 intravenously through the tail vein with 8 μ g of FAM-VAD-FMK. The reagent was allowed to circulate in the mouse for 30 minutes before photographing with 488 nm excitation. The results demonstrate a higher level of apoptosis.

Figure 7. Each mouse was injected with 2×10^5 SCK tumor cells. The tumors were allowed to grow for 7 days. The control mouse was injected with a placebo and the test mouse was injected with 8.0 mg/kg of ATO. After 24 hours
15 of treatment the mice were injected intravenously through the tail vein with 8 μ g of FAM-VAD-FMK. The reagent was allowed to circulate in the mouse for 30 minutes before excising the tumors. The tumors were then broken apart and the cells were dispersed. The cells were then analyzed by flow cytometry. Flow
20 cytometry analysis demonstrated that the treated mouse had an apoptosis induction rate of 39% while the control mouse had an apoptosis induction rate of only 18%.

Figure 8. Apoptosis imaging of FSaII tumor in DSFC in a nu/nu mouse. 10X capture of apoptosis prior to ATO injection, 45 minutes after 12.0 μ g of
25 FAM-VAD-FMK were injected intravenously through the tail vein. Very little apoptosis is visible.

Figure 9. Same tumor as in Figure 8. 10X capture of apoptosis 3 hours post ATO (8.0 mg/kg) injection. 45 minutes after 12.0 μ g of FAM-VAD-FMK were injected intravenously through the tail vein. The increase in apoptosis
30 within the tumor is clear.

Summary of Certain Embodiments of the Invention

Accordingly, certain embodiments of the invention provide methods and products, such as kits, useful for determining the apoptotic state of cells *in vivo*, such as in an organism such as an animal.

5 This invention provides cell permeant probes that bind specifically to up-regulated caspase and apoptosis-associated enzymes, which allows for the detection and imaging of apoptosis events *in vivo*.

Certain embodiments of the invention provide a method for *in vivo* determination of the apoptotic state of one or more cells in an animal, including
10 detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the presence or abundance of the caspase affinity labeling agent correlates with the apoptotic state of the cells.

Certain embodiments of the invention provide a method for *in vivo*
15 determination of whether a therapeutic agent modulates apoptosis in one or more cells in an animal, including detecting the presence of at least one caspase affinity labeling agent in the cells of an animal that has been previously treated with the therapeutic agent and into which at least one caspase affinity labeling agent has been introduced, wherein the presence of the caspase affinity labeling
20 agent correlates with the ability of the therapeutic agent to modulate apoptosis. In certain embodiments of the invention, the method determines whether the therapeutic agent increases or decreases apoptosis.

Certain embodiments of the invention provide a method for *in vivo* determination of whether a radiation treatment modulates apoptosis in one or
25 more cells in an animal, including detecting the presence of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the animal has been previously treated with radiation, wherein the presence of the caspase affinity labeling agent correlates with the ability of the radiation to modulate apoptosis.
30 In certain embodiments of the invention, the method determines whether the radiation treatment increases or decreases apoptosis.

Certain embodiments of the invention provide an *in vivo* diagnostic method for determining the presence or absence of a disease characterized by the presence of apoptosis including detecting the presence of at least one caspase affinity labeling agent in the cells of the animal into which at least one caspase
5 affinity labeling agent has been introduced, wherein the presence or absence of the at least one caspase affinity labeling agent correlates with the presence or absence of the disease.

In some embodiments of the invention, the diseases include but are not limited to eye disease such as glaucoma, retinal diseases such as macular
10 degeneration and proliferative retinopathy; neurodegenerative diseases such as neuropathy, Alzheimer's disease, multiple sclerosis, Huntington's disease and others; and cardiac disease.

Certain embodiments of the invention provide an *in vivo* method for evaluating the sensitivity of a disease to at least one therapeutic agent or
15 treatment, including detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the animal has previously received the therapeutic agent or treatment, wherein the presence or abundance of the caspase affinity labeling agent correlates with the sensitivity of the disease
20 to the at least one therapeutic agent or treatment.

Certain embodiments of the invention provide a method for the monitoring of cancer treatment in an animal, including: detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of the animal into which at least one caspase affinity labeling agent has been introduced that
25 has received a therapeutic agent or treatment, wherein the presence or abundance of at least one caspase affinity labeling agent correlates with the efficacy of the therapeutic agent or treatment.

Certain embodiments of the invention provide a method for the monitoring of leukemia treatment in an animal, including detecting the presence
30 or abundance of at least one caspase affinity labeling agent in the cells of the animal into which at least one caspase affinity labeling agent has been introduced that has received a therapeutic agent or treatment, wherein the presence or

abundance of at least one caspase affinity labeling agent correlates with the efficacy of the therapeutic agent or treatment.

Certain embodiments of the invention provide a method for the monitoring of blood and bone marrow disease treatment in an animal including
5 detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced that has received a therapeutic agent or treatment, wherein the presence or abundance of at least one caspase affinity labeling agent correlates with the efficacy of the therapeutic agent or treatment.

10 Certain embodiments of the invention provide a method for determining if one or more compounds within a chemical library modulate caspase activity in an animal including determining the level of at least one caspase affinity labeling agent in cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the animal has been contacted with one or more
15 compounds from the library, and determining whether the one or more compounds modulate the caspase activity.

Certain embodiments of the invention provide a method for determining if one or more compounds within a chemical library modulate apoptosis in an animal including determining the level of at least one caspase affinity labeling
20 agent in cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the animal has been contacted with one or more compounds from the library, and determining whether the one or more compounds modulate apoptosis.

In certain embodiments of the invention, the determination step includes
25 comparing the level of affinity labeling agent in the animal with a control animal not exposed to the compound.

In certain embodiments, the determination step is a longitudinal study that is comprised of a comparison of the level of affinity labeling agent in the animal before exposure to the compound and after the animal has been exposed
30 to the compound. In this case the animal is contacted with the affinity labeling agent before exposure to the compound and a second time, after exposure to the compound.

In certain embodiments of the invention, detection is carried out using NMR, MRI, CT, CAT, or PET scans; a flow cytometer; a laser scanning cytometer; a fluorescence microplate reader; a luminescence microplate reader, a chromogenic microplate reader; a fluorescence microscope; a confocal
5 microscope; a luminescence microscope, or scintigraphy; a bright-field microscope; a whole animal fluorescence imaging systems (optical imaging system); or a whole animal luminescence imaging system, or a combination thereof.

In certain embodiments of the invention, detection is carried out using a
10 Window Chamber inserted into the animal.

In certain embodiments of the invention, detection is carried out using a fluorescence microscope; a confocal microscope; a bright-field microscope, or luminescence microscope.

In certain embodiments of the invention, detection is carried out or
15 confirmed by removing a sample from the animal such as by extraction, biopsy, venipuncture, dissection, or other suitable methods and detection is carried out on a sample that has been removed from the animal.

In certain embodiments of the invention, detection is via a flow cytometer; a laser scanning cytometer; a fluorescence microplate reader; a
20 chromogenic microplate reader; a fluorescence microscope; a confocal microscope; a bright-field microscope; a luminescence microplate reader; or a luminescence microscope.

In certain embodiments of the invention, the presence or abundance of the affinity labeling agent is detected in the bone marrow, thymus, lymph nodes,
25 spleen or circulating blood of the animal.

In certain embodiments of the invention, the presence or abundance of the affinity labeling agent is detected in peripheral blood monocytes (PBMCs).

In certain embodiments of the invention, the cells are included in tissues, organs or tumors of the animal.

30 In certain embodiments of the invention, the caspase affinity labeling agent is introduced into the animal by intravenous, intravascular, intraperitoneal,

intravitreal, intraocular, intracranial, intrapleural, intrathoracic, intramuscular, intrapulmonary, injection, perfusion, or lavage administration.

As used herein, the term animal refers to any type of living organism, *e.g.*, a multi-cellular organism. In certain embodiments of the invention, the animal is a mammal. In certain embodiments of the invention, the mammal is a human male or female.

Certain embodiments of the invention provide an assay kit including packaging materials and one or more caspase affinity labeling agents and instructions for using the caspase affinity labeling agents to determine the level of apoptosis *in vivo*.

In certain embodiments of the invention, the caspase affinity labeling agent is a cell permeant probe consisting of a compound of formula I:



wherein:

L_1 is a detectable group that may comprise gadolinium (Gd), Terbium (Tb), Europium (Eu) or any other Lanthanide series element (*e.g.*, Ce, Pr, Nd, Pm, Sm, Dy, Ho, Er, Tm, Yb, or Lu) or Iron (Fe), Manganese (Mn), Rhenium (Re), or Technetium (Tc). The detectable group may be suitable for nuclear magnetic resonance (NMR) or magnetic resonance imaging (MRI) detection, or luminescence or scintigraphy. The detectable group may be iodine (I) or barium (Ba), *e.g.*, for computer tomography (CT scan) or computer axial tomography (CAT scan) detection. The detectable group may be a positron emitter (such as ^{11}C , ^{13}N , ^{15}O or ^{64}Cu), *e.g.*, for positron emission tomography (PET scan). The detectable group may be a fluorescent label (*e.g.*, a fluorescein derivative, sulforhodamine derivative, Cy dye derivative, BODIPY derivative, coumarin derivative, Quantum Dot, or any fluorescent dye that can be attached, *e.g.*, to an amino group, directly or by linkers). The detectable group may be a radioisotope (*e.g.* 3H , ^{14}C , or ^{35}S).

A_1 is a direct bond or a linker that can simply be a covalent bond. The detectable group may be attached directly, *e.g.*, to the N-terminal amino group of the peptide or amino acid (*e.g.*, amide linkage $L-(C=O)-NH-R$). A_1 can also be

any member of the class of linkers well known to the art. Linkers are typically about 4-18 atoms long, including carbon, nitrogen, oxygen or sulfur atoms;

X_1 is absent, an amino acid, or a peptide which may be a peptide having about 1 to 10 amino acids, *e.g.*, about 2 to 4 amino acids (*e.g.*, V, VA, YVA, DEV, LEE, LEH, VDVA, IET, WHE or AEV). For a compound of formula (I), X_1 may be a natural amino acid (*e.g.*, A, V, or E). For a compound of formula (I), R_1' may be a methylene carboxy (ethanoic) side-chain (CH_2-COOH) as caspases typically have an aspartate in the P_1 position of the peptide substrate.

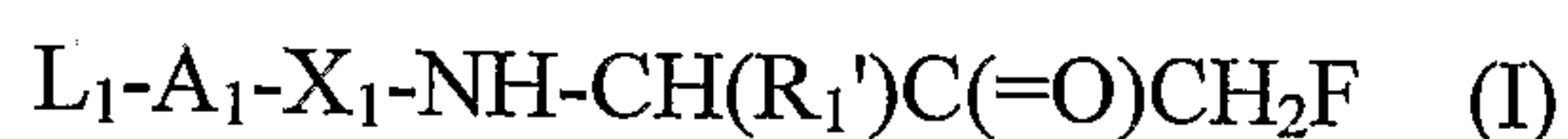
R_1' may be an aspartic acid side-chain (CH_2-COOH) or an ester of aspartic acid (*e.g.*, $-CH_2CO_2R$, where R is C_1-C_6 alkyl or benzyl, CH_3 , C_2H_5 or $CH_2C_6H_5$), for example. Certain caspase affinity labeling probes may contain the same labels and a 1 to 5 amino acid sequence, but utilize an aza-peptide epoxide modification of the aspartic acid (*see, e.g.*, U.S. Patent No. 7,056,947 B2), or an aza-peptide Michael acceptor (Ekici *et al.*, 2004), an aldehyde modification of the aspartic terminal carboxyl group ($HC=O$), a chloromethyl ketone group (CH_2Cl), or an acyloxy reactive group ($(C=O)O-Ar$, where Ar is [2,6-(CF_3)₂]benzoate or various derivative of same (Krantz *et al.*, 1991, and Thornberry *et al.*, 1994).

Detailed Description

Certain embodiments of the invention provide methods and products, such as kits, useful for determining the apoptotic state of cells in an organism such as a human.

In some embodiments, the invention provides a method for *in vivo* determination of the apoptotic state of one or more cells (*e.g.*, viable whole cells), which may, *e.g.*, be included in tissues, organs or tumors, in mammals such as humans, including: 1) contacting the cells *in vivo* with at least one caspase affinity labeling agent that is introduced into the subject, *e.g.*, by intravenous, intravascular, intraperitoneal, intravitreal, intraocular, intracranial, intrapleural, intrathoracic, intramuscular, intrapulmonary, injection, perfusion, or lavage administration; and 2) detecting the presence or abundance of at least one affinity labeling agent in the cells; wherein the presence or abundance of the caspase affinity labeling agent correlates with the apoptotic state of the cells.

In some embodiments of the invention, the caspase affinity labeling agent is a cell permeant probe. It is believed that the caspase affinity labeling agent may inhibit active caspases by binding covalently to the active catalytic site and is retained within the cell. It is believed that these labeled membrane permeant probes penetrate the cell membrane of live cells and covalently bind to active caspase enzymes in apoptotic cells, thereby allowing for specific and sensitive detection of apoptosis (Bedner *et al.*, 2000, Smolewski *et al.*, 2001, and Smolewski *et al.*, 2002). In certain embodiments of the invention, the caspase affinity labeling agent is a compound of formula I:



wherein:

L_1 is a detectable group that may comprise gadolinium (Gd), Terbium (Tb), Europium (Eu) or any other Lanthanide series element (*e.g.*, Ce, Pr, Nd, Pm, Sm, Dy, Ho, Er, Tm, Yb, or Lu) or Iron (Fe), Manganese (Mn), Rhenium (Re), or Technetium (Tc). The detectable group may be suitable for nuclear magnetic resonance (NMR) or magnetic resonance imaging (MRI) detection, or luminescence or scintigraphy. The detectable group may be iodine (I) or barium (Ba), *e.g.*, for computer tomography (CT scan) and computer axial tomography (CAT scan) detection. The detectable group may be a positron emitter (such as ^{11}C , ^{13}N , ^{15}O , or ^{64}Cu), *e.g.*, for positron emission tomography (PET scan). The detectable group may be a fluorescent label (*e.g.*, a fluorescein derivative, sulforhodamine derivative, Cy dye derivative, BODIPY derivative, coumarin derivative, Quantum Dot, or any fluorescent dye that can be attached, *e.g.*, to an amino group, directly or by linkers. The detectable group may be a radioisotope (*e.g.* 3H , ^{14}C , ^{35}S).

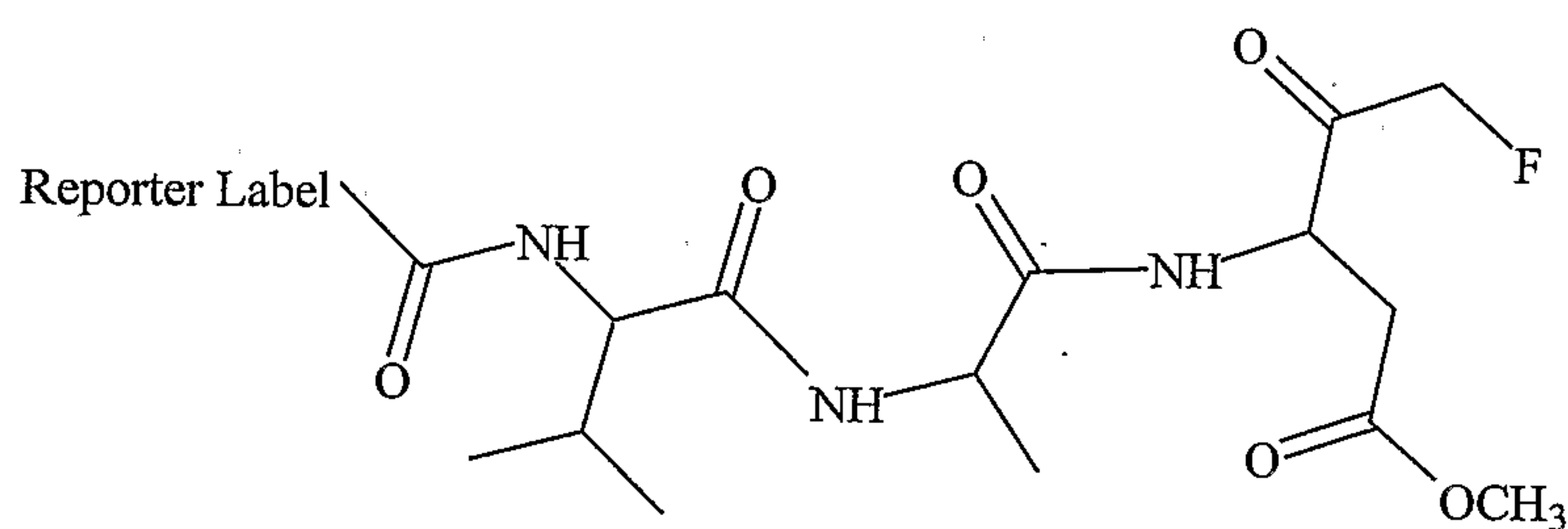
A_1 is a direct bond or a linker that can simply be a covalent bond. The detectable group may be attached directly, *e.g.*, to the N-terminal amino group of the peptide or amino acid (*e.g.*, amide linkage $L-(C=O)-NH-R$). A_1 can also be any member of the class of linkers well known to the art. Linkers are typically about 4-18 atoms long, including carbon, nitrogen, oxygen or sulfur atoms.

X_1 is absent, an amino acid, or a peptide which may be a peptide having about 1 to 10 amino acids, *e.g.*, about 2 to 4 amino acids (*e.g.*, V, VA, YVA, DEV, LEE, LEH, VDVA, IET, WHE or AEV). For a compound of formula (I), X_1 may be a natural amino acid (*e.g.*, A, V, or E). For a compound of formula
 5 (I), R_1' may be a methylene carboxy (ethanoic) side-chain ($\text{CH}_2\text{-COOH}$) as caspases typically have an aspartate in the P_1 position of the peptide substrate.

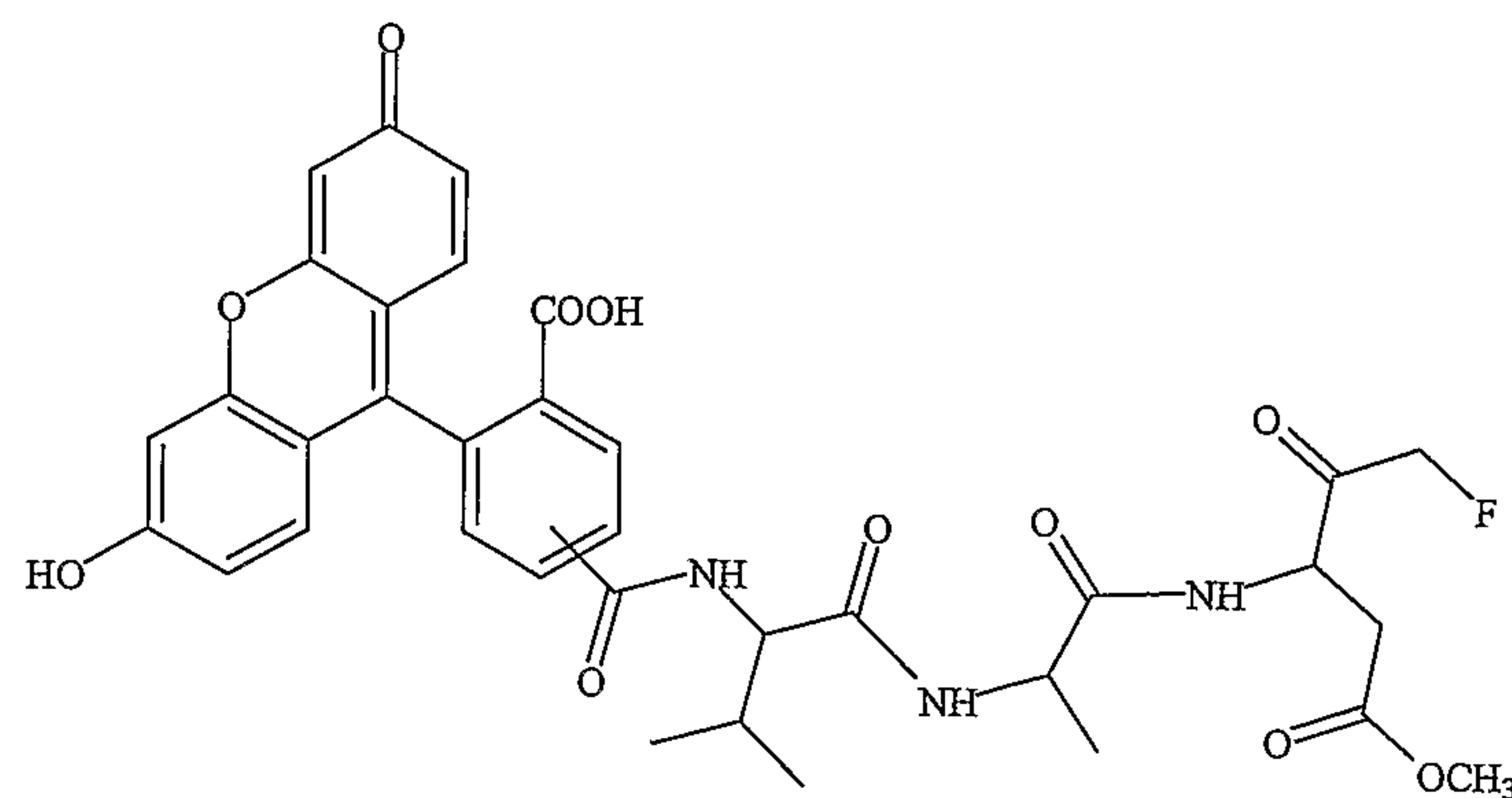
R_1' may be an aspartic acid side-chain ($\text{CH}_2\text{-COOH}$) or an ester of aspartic acid (*e.g.*, $\text{-CH}_2\text{CO}_2\text{R}$, where R is $\text{C}_1\text{-C}_6$ alkyl or benzyl, CH_3 , C_2H_5 or $\text{CH}_2\text{C}_6\text{H}_5$), for example. Certain caspase affinity labeling probes may contain the
 10 same labels and a 1 to 5 amino acid sequence, but utilize an aza-peptide epoxide modification of the aspartic acid (*see, e.g.*, U.S. Patent No. 7,056,947 B2), or an aza-peptide Michael acceptor (Ekici *et al.*, 2004), an aldehyde modification of the aspartic terminal carboxyl group (HC=O), a chloromethyl ketone group (CH_2Cl), or an acyloxy reactive group ($(\text{C=O})\text{O-Ar}$, where Ar is [2,6-
 15 $(\text{CF}_3)_2$]benzoate or various derivative of same (Krantz *et al.*, 1991, and Thornberry *et al.*, 1994).

The following structures are examples of compounds of formula I, but the invention is not limited to these structures. The term "reporter label" is used interchangeably with "detectable group".

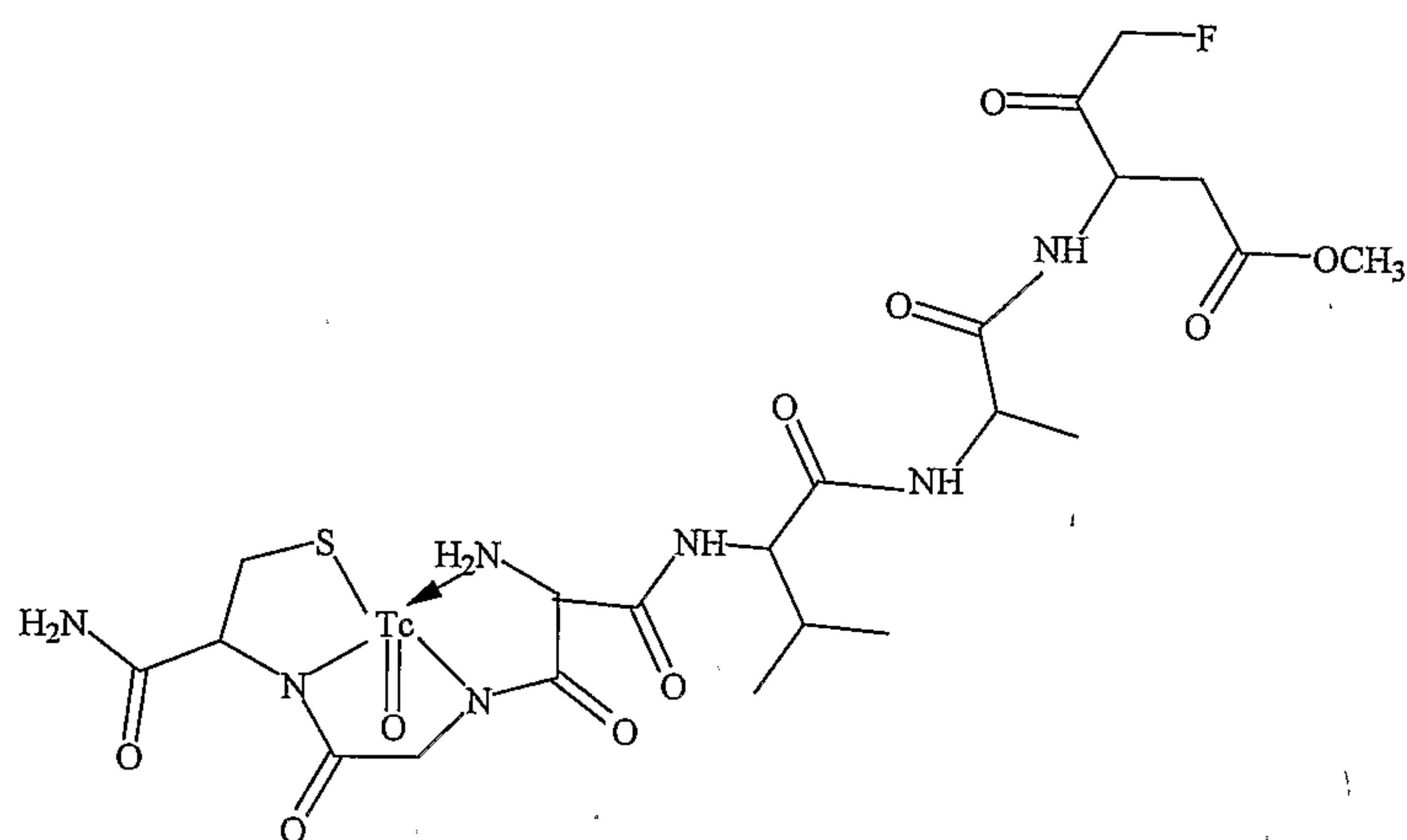
20 One example is an Asp(OMe)-FMK modified reactive end that is believed to bind to the cysteine residue in the catalytic site of active caspases.



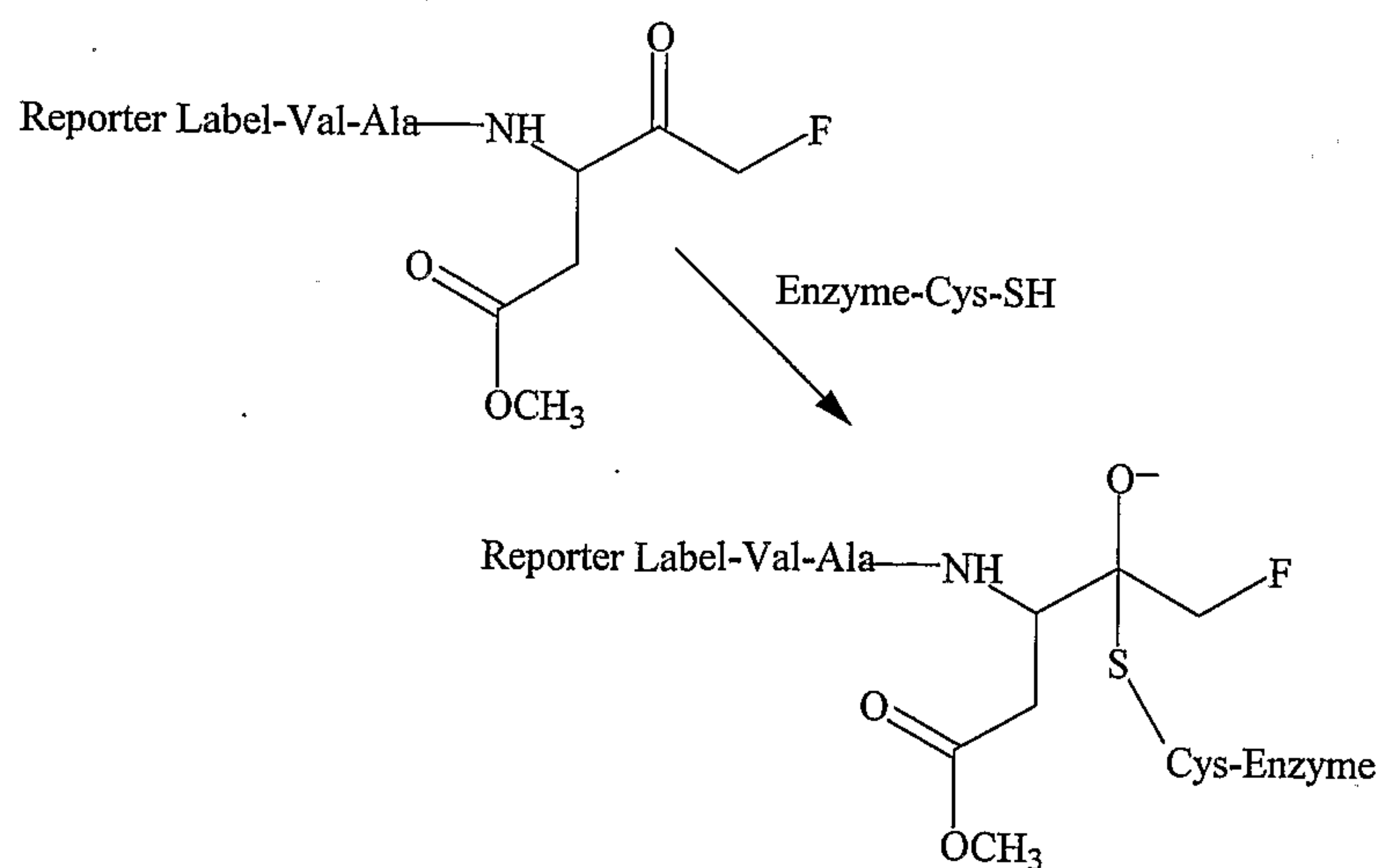
One example of a fluorescent labeled FMK caspase ligand is a carboxyfluorescein-valanyl-alanyl-aspartyl(O-methyl)-fluoromethyl ketone.



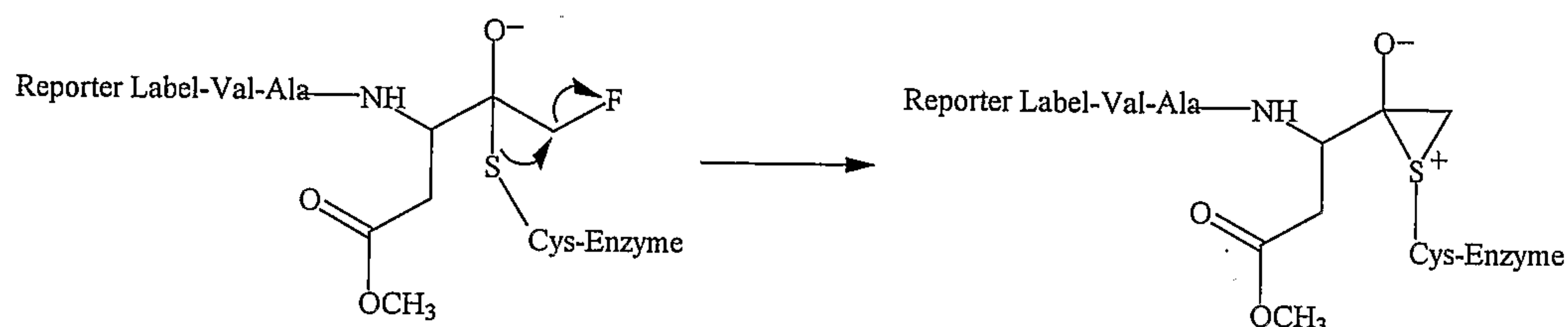
One example of a metal chelate labeled FMK caspase ligand is a Tc-valanyl-alanyl-aspartyl(O-methyl)-fluoromethyl ketone.



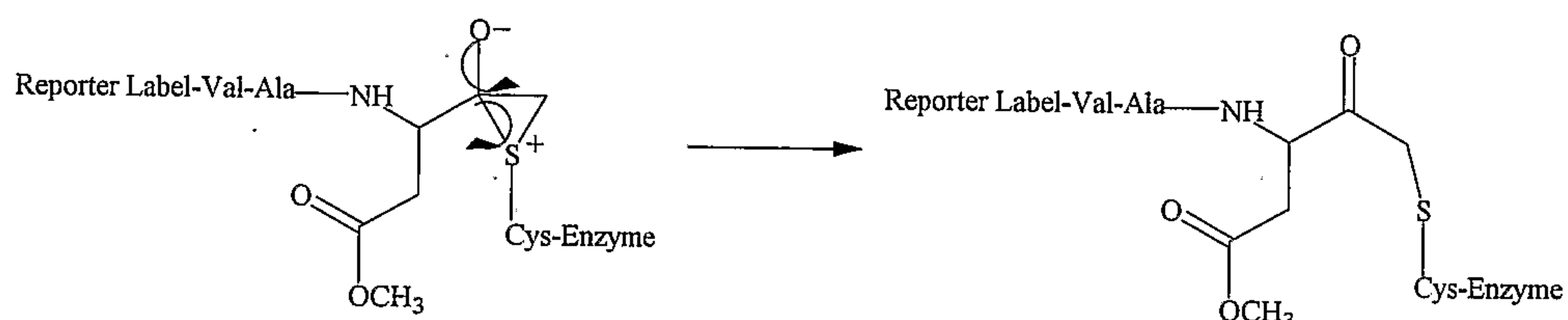
- 5 Once the fluoromethyl ketone peptides enter the cell and come into contact with an active caspase, it is believed that the sequence is recognized by the catalytic site and forms a covalent bond through a three step process. In the first step it is believed that the formation of a thiohemiketal intermediate occurs.



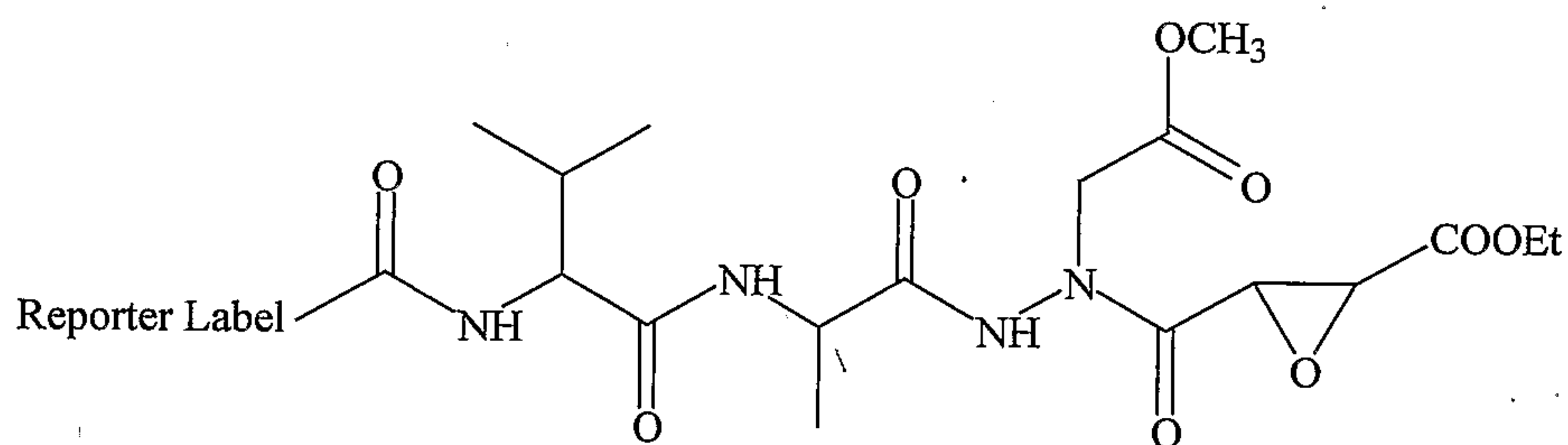
It is believed that the thiohemiketal intermediate undergoes a rearrangement to form a three membered sulfonium intermediate.



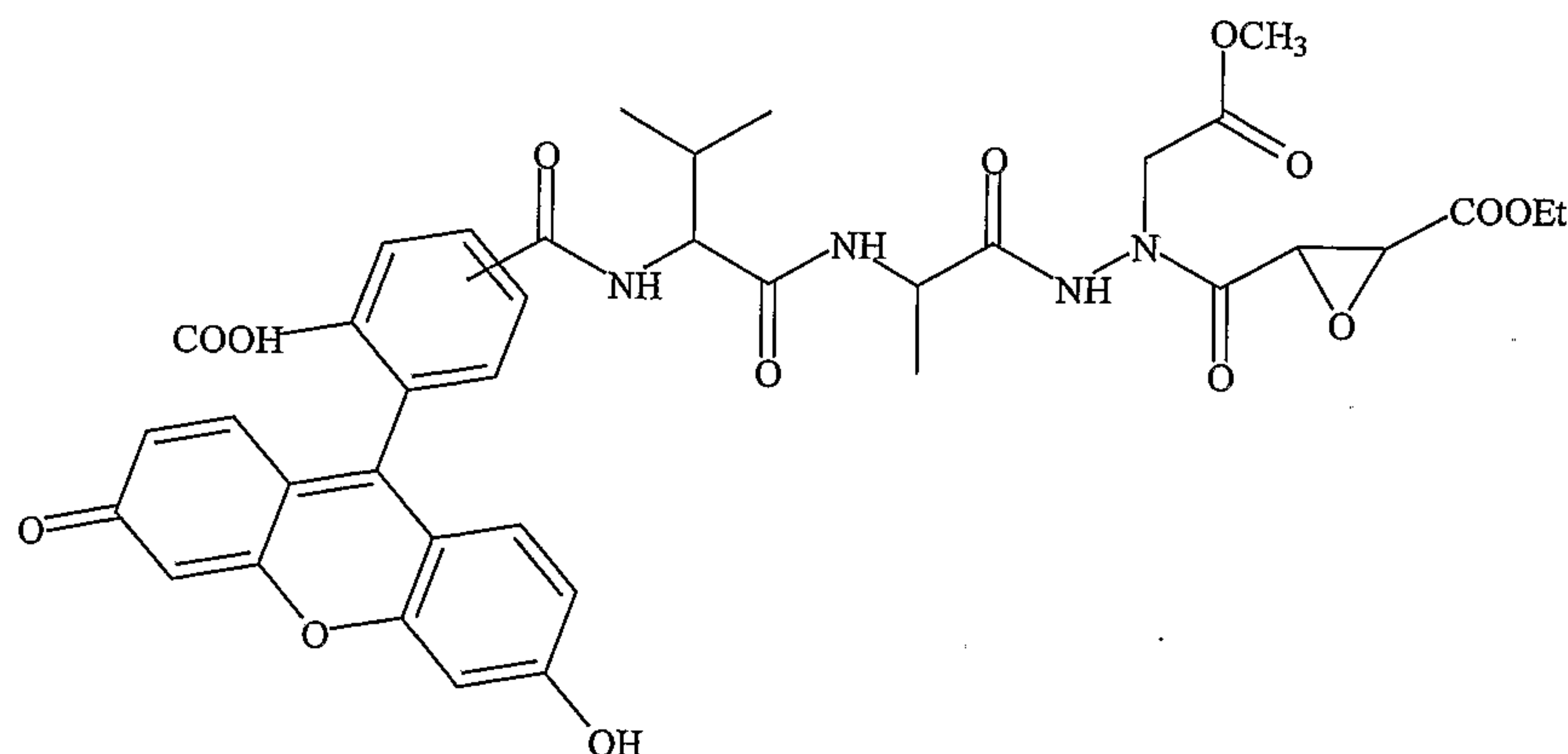
It is believed that the second intermediate rearranges to give the final stable thioether adduct, which is retained within the apoptotic cell.



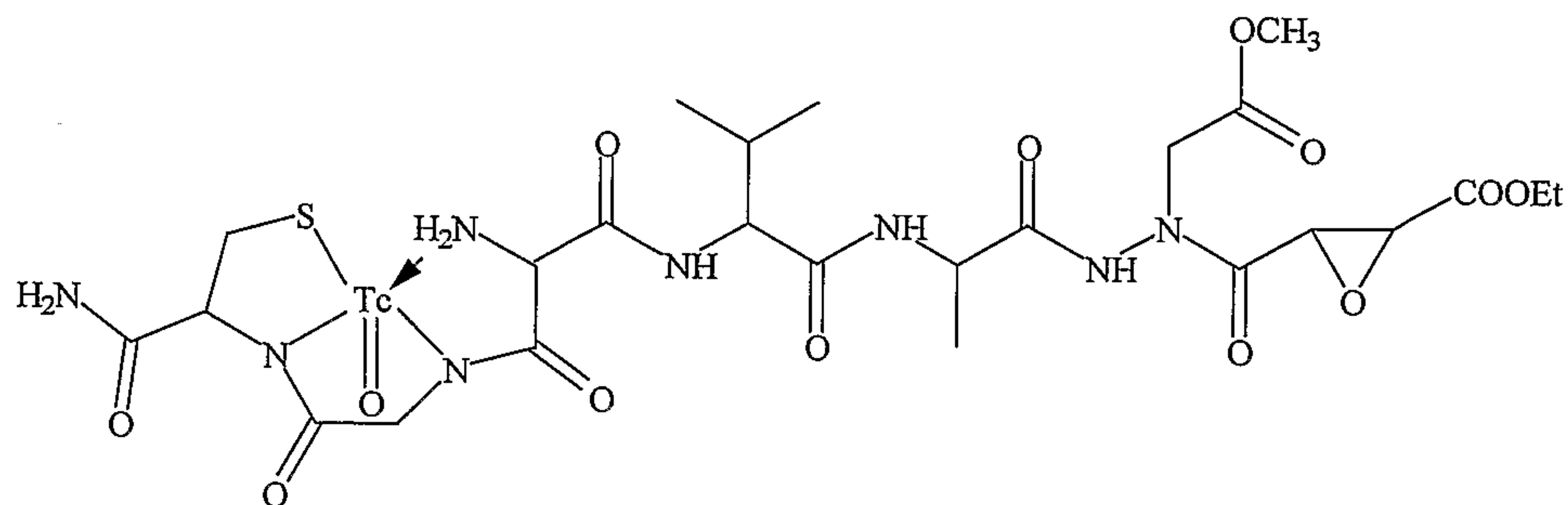
The following structure is an example of an aza-peptide epoxide modification of the reactive end that is believed to bind to the cysteine residue in the catalytic site of active caspases.



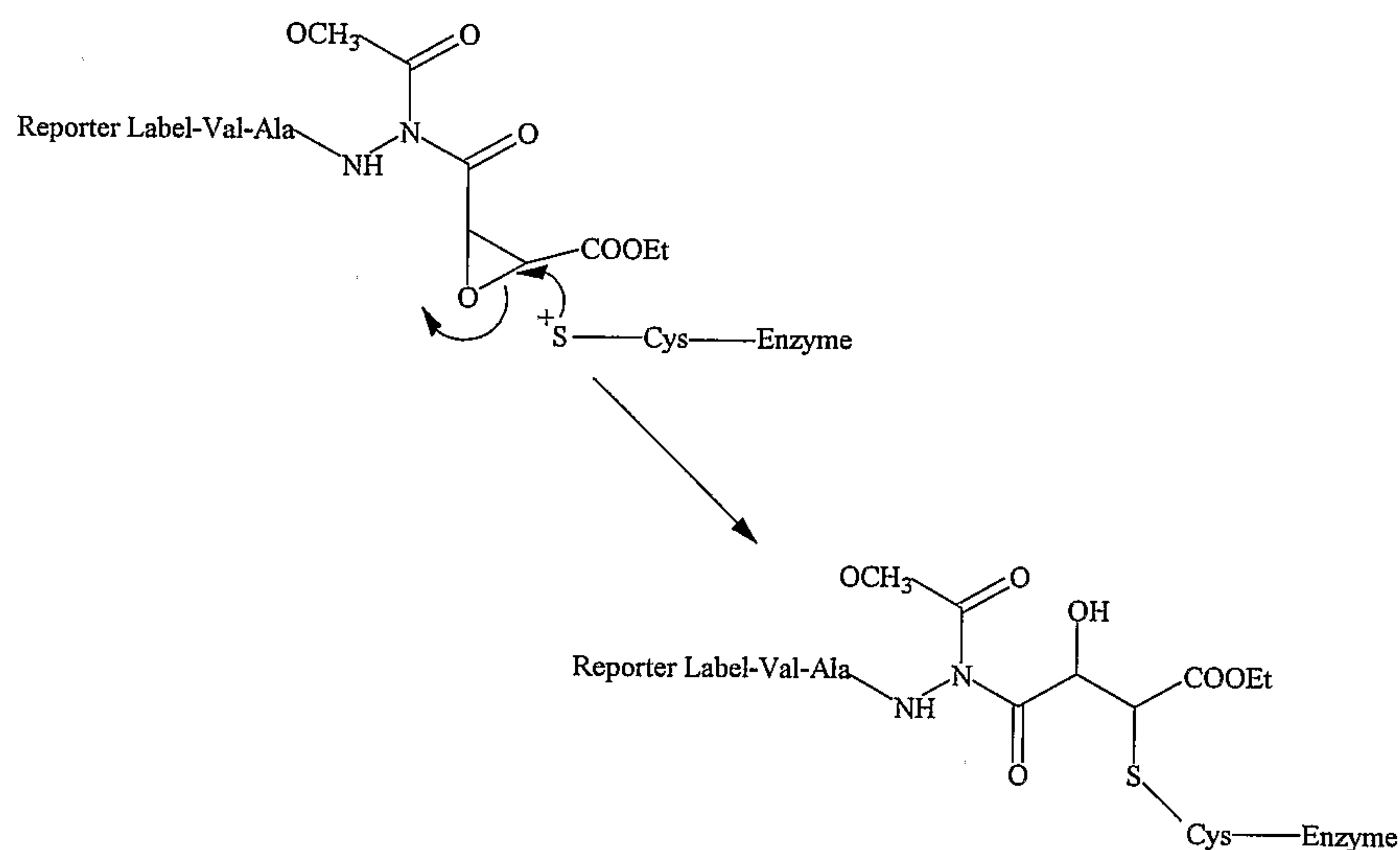
One example of a fluorescent labeled aza-peptide epoxide modified caspase ligand is a carboxyfluorescein-valanyl-alanyl-_{aza}aspartyl(O-methyl)-Epoxide.



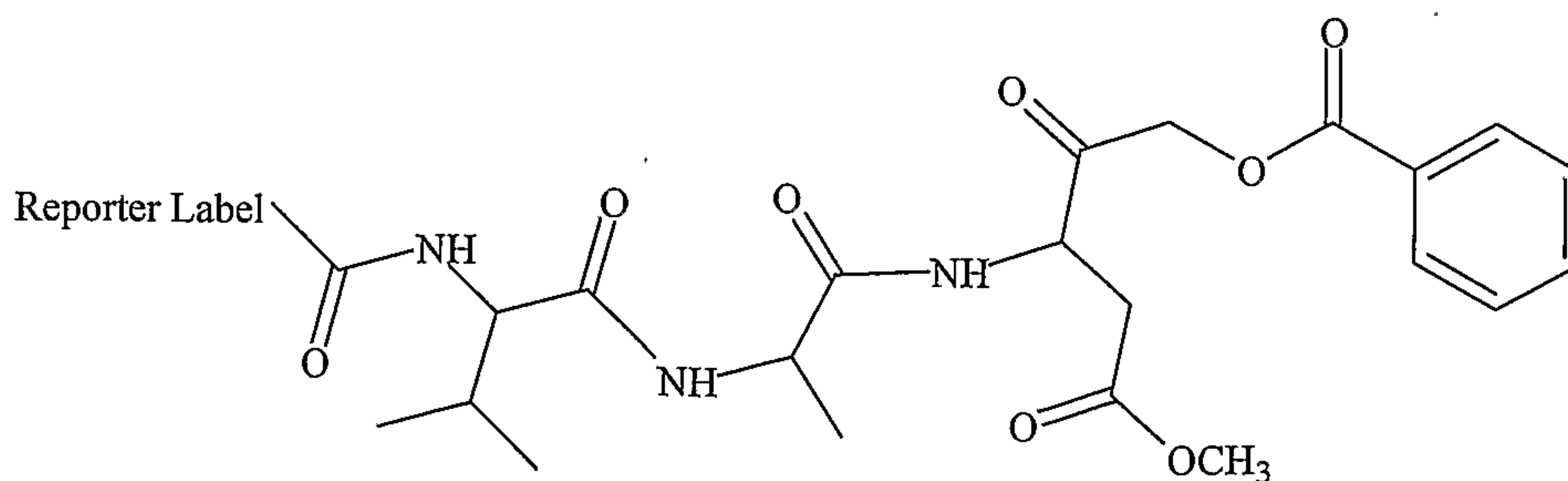
One example of a metal chelate labeled aza-peptide epoxide modified caspase ligand is a Tc-valanyl-alanyl-_{aza}aspartyl(O-methyl)-Epoxide.



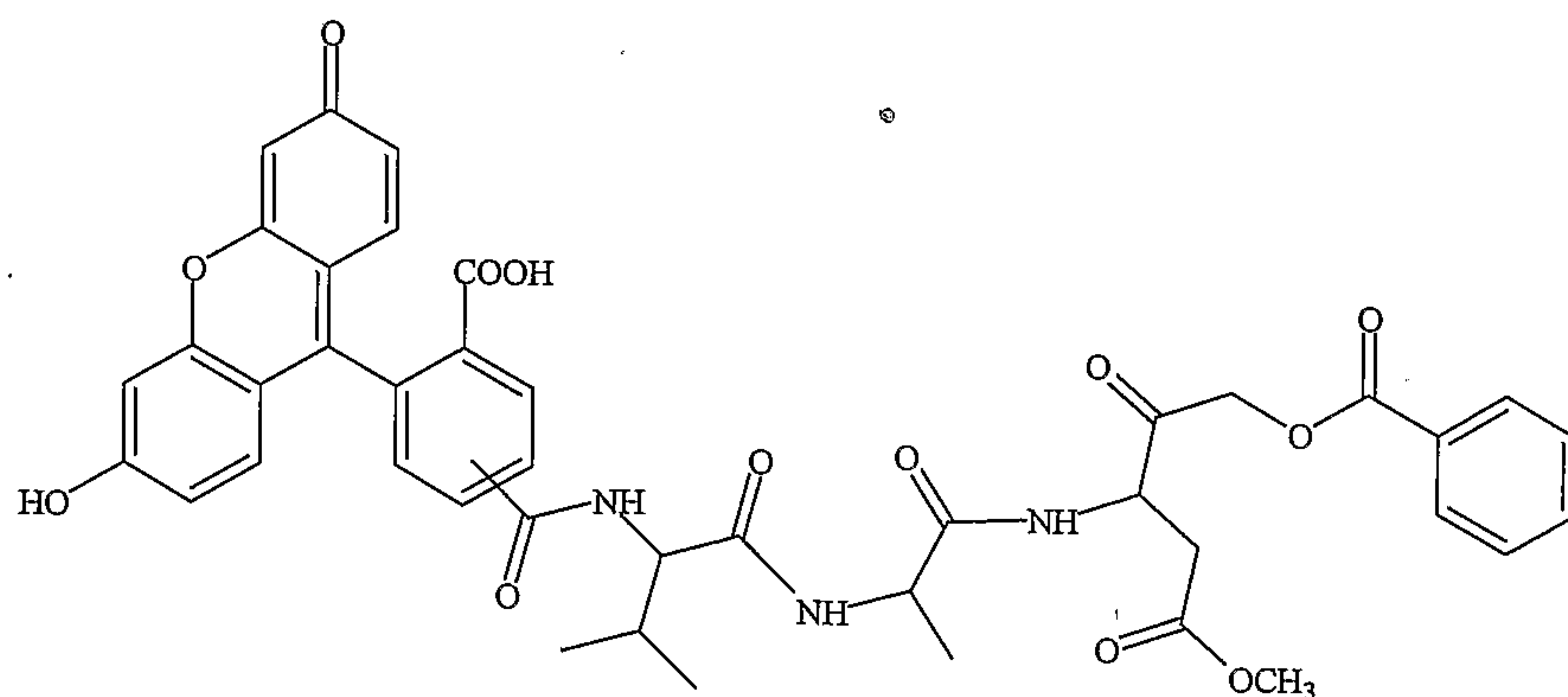
It is believed that once the aza-peptide epoxide modified peptides enter
5 the cell and come into contact with an active caspase, the sequence is recognized
by the catalytic site and forms a covalent bond where the active site cysteine
nucleophilically attacks the three-member ring forming the final product that will
remain in the apoptotic cell.



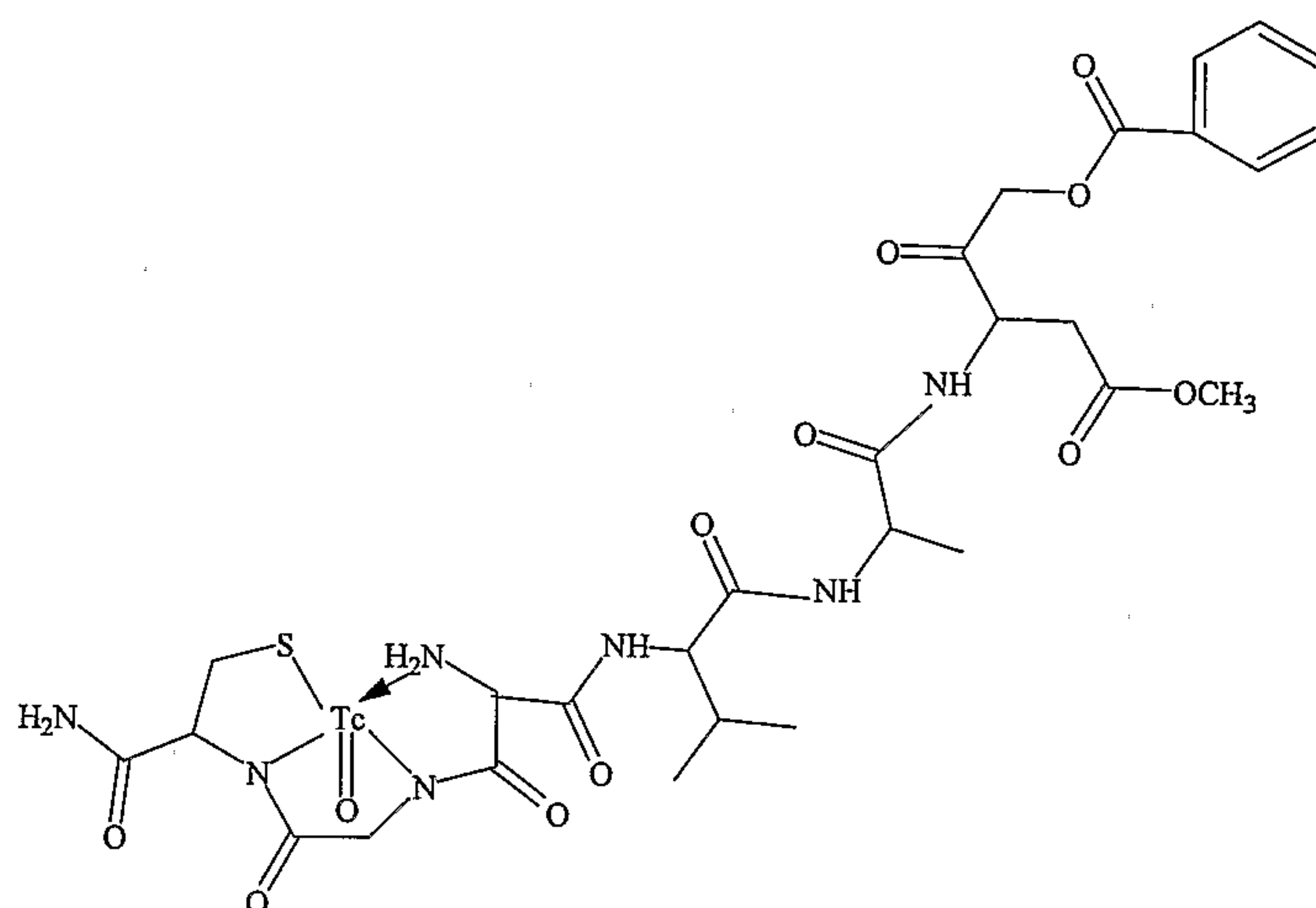
10 An Asp(OMe)-OPh modified reactive end is believed to bind to the
cysteine residue in the catalytic site of active caspases.



One example of a fluorescent labeled OPh caspase ligand is carboxyfluorescein-valanyl-alanyl-aspartyl(O-methyl)-benzoyloxymethyl ketone.



One example of a metal chelate labeled OPh caspase ligand is Tc-valanyl-alanyl-aspartyl(O-methyl)-benzoyloxymethyl ketone.



- 5 Following synthesis, the agent may be further processed into a vial by dissolving it in a suitable organic solvent such as acetone, acetonitrile, dimethylformamide (DMF), dimethylsulfoxide (DMSO), 1,4-dioxane or tetrahydrofuran. Once dissolved, the agent may then be diluted into a suitable non-aqueous or aqueous liquid and dispensed into individual vials. The
- 10 individual vials may be cooled to less than 0°C, lyophilized under vacuum between 10 to 500 millitor to dryness at -80°C to +100°C, with or without a liquid nitrogen or dry ice trap. Individual vials can then be capped and stored between -80°C and 20°C.

- The *in vivo* apoptosis detection and imaging kits may include one or
- 15 more vials of reagent (*e.g.*, processed and lyophilized reagent) and an appropriate Injection Buffer (*e.g.*, a bottle of 10X Injection Buffer). A 10X Injection Buffer may be prepared according to the following recipe in endotoxin free DI H₂O:

87.66 g/L of NaCl (1.5 M), 60.53 g/L of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ + 4.84 g/L of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.2 M phosphate), pH 6.9.

Prior to use, the reagent may be dissolved in DMSO and may be used immediately or aliquoted and stored, *e.g.*, at less than -20°C . The 10X Injection Buffer may be diluted 1:10 in endotoxin free DI H_2O and filter sterilized resulting in a final pH of 7.4. The reagent may be diluted to injection concentrations in the sterilized 1X Injection Buffer. The reagent may be injected into the animal between 0.1 micromoles per kg and 1.0 milimoles per kg. It may then be allowed to circulate throughout the animal, *e.g.*, for from about 5 minutes to several hours before detection and imaging of apoptosis.

Certain embodiments of the invention provide a method for *in vivo* determination of whether a therapeutic agent induces apoptosis in one or more viable whole cells, tissues, organs or tumors in mammals, including humans, including: 1) treating the subject with the therapeutic agent; 2) introducing a caspase affinity labeling agent into the subject; and 3) detecting the presence of the group L in the cells, tissues, organs, or tumors, wherein the presence of L correlates with the ability of the agent to induce apoptosis.

Certain embodiments of the present invention also provide a method for *in vivo* determination of whether a therapeutic agent reduces or inhibits apoptosis in one or more viable whole cells, tissues, organs, or tumors in mammals, including humans, including: 1) treating the subject with the therapeutic agent; 2) introducing a caspase affinity labeling agent into the subject; and 3) detecting the presence of the group L in the cells, tissues, organs, or tumors, wherein the presence of L correlates in a negative sense, with whether the therapeutic agent reduces or inhibits apoptosis.

Certain embodiments of the present invention also provide a method for *in vivo* determination of whether a radiation treatment induces apoptosis in one or more viable whole cells, tissues, organs, or tumors in mammals, including humans, including: 1) treating the subject with radiation; 2) introducing a caspase affinity labeling agent into the subject; and 3) detecting the presence of the group L in the cells, tissues, organs, or tumors, wherein the presence of L correlates with the ability of the agent to induce apoptosis.

Certain embodiments of the invention provide a method or *in vivo* determination of whether a radiation reduces or inhibits apoptosis in one or more viable whole cells, tissues, organs, or tumors in mammals, including humans, including: 1) treating the subject with the radiation; 2) introducing a caspase
5 affinity labeling agent into the subject; and 3) detecting the presence of the group L in the cells, tissues, organs, or tumors, wherein the presence of L correlates in a negative sense, with whether the therapeutic agent reduces or inhibits apoptosis.

Certain embodiments of the invention provide an *in vivo* diagnostic method for determining the presence or absence of a disease characterized by the
10 presence of apoptosis in one or more viable whole cells, tissues, organs, or tumors in mammals, including humans, including: 1) introducing a caspase affinity labeling agent into the subject; and 2) detecting the presence of the group L in the cells, tissues, organs, or tumors; wherein the presence of L correlates with the presence or absence of the disease.

15 Certain embodiments of the invention provide an *in vivo* diagnostic method to diagnose macular degeneration and proliferative retinopathy by determining the presence or absence of a disease characterized by the presence of apoptosis in one or more viable whole cells within the retina in mammals, including humans, including: 1) introducing a caspase affinity labeling agent
20 into the subject; and 2) detecting the presence of the group L in the retina; wherein the presence of L correlates with the presence or absence of the disease.

Certain embodiments of the invention provide an *in vivo* diagnostic method to diagnose glaucoma by determining the presence or absence of a disease characterized by the presence of apoptosis in one or more viable retinal
25 glial cells in mammals, including humans, including: 1) introducing a caspase affinity labeling agent into the subject; and 2) detecting the presence of the group L in the retinal glial cells; wherein the presence of L correlates with the presence or absence of the disease.

Certain embodiments of the invention provide an *in vivo* diagnostic
30 method to assess the amount of damage in cardiac disease by determining the presence or absence of a disease characterized by the presence of apoptosis in one or more viable whole cells within cardiac tissue in mammals, including

humans, including: 1) introducing a caspase affinity labeling agent into the subject; and 2) detecting the presence of the group L in the cardiac tissues; wherein the presence of L correlates with the presence or absence of the disease.

Certain embodiments of the invention provide an *in vivo* diagnostic method to assess the amount of damage in neurodegenerative disease by determining the presence or absence of a disease characterized by the presence of apoptosis in one or more viable whole cells and neurons within nerve tissue and the brain in mammals, including humans, including: 1) introducing a caspase affinity labeling agent into the subject; and 2) detecting the presence of the group L in the nerve tissues; wherein the presence of L correlates with the presence or absence of the disease.

Certain embodiments of the invention provide an *in vivo* method for evaluating the sensitivity of a disease in a mammal to a therapeutic agent or treatment, wherein the presence- or level of apoptotic activity correlates with the sensitivity of the disease in mammals, including humans, including: 1) subjecting the mammal to the therapeutic agent or treatment, 2) introducing a caspase affinity labeling agent into the subject; and 3) detecting the presence or abundance of the affinity labeling agent in the cells, tissues, organs, or tumors; wherein the presence or abundance correlates with the sensitivity.

Certain embodiments of the invention provide a method for the monitoring of cancer treatment, wherein the presence- or level of apoptotic activity correlates with the efficacy of the treatment in mammals, including humans, including: 1) subjecting the subject to the therapeutic agent or treatment; 2) introducing a caspase affinity labeling agent into the subject; and 3) detecting the presence or abundance of each of the affinity labeling agent in the cells, tissues, organs, or tumors; wherein the presence or abundance of apoptosis correlates with efficacy.

Certain embodiments of the invention provide a method for the monitoring of leukemia treatment, wherein the presence- or level of apoptotic activity correlates with the efficacy of the treatment in mammals, including humans, including: 1) subjecting the subject to the therapeutic agent or treatment; 2) introducing a caspase affinity labeling agent into the subject; and 3)

detecting the presence or abundance of the affinity labeling agent in the bone marrow, thymus, lymph nodes, spleen and circulating blood; wherein the presence or abundance of apoptosis correlates with efficacy.

Certain embodiments of the invention provide a method for the
5 monitoring of blood and bone marrow disease treatment, wherein the presence-
or level of apoptotic activity correlates with the efficacy of the treatment in
mammals, including humans, including: 1) subjecting the subject to the
therapeutic agent or treatment; 2) introducing a caspase affinity labeling agent
into the subject; 3) drawing blood and preparing peripheral blood monocytes
10 (PBMCs); and 4) detecting the presence or abundance of the affinity labeling
agent in the cells; wherein the presence or abundance of apoptosis correlates with
treatment efficacy.

Certain embodiments of the invention provide a method for the
monitoring of leukemia treatment, wherein the presence- or level of apoptotic
15 activity correlates with the efficacy of the treatment, in mammals, including
humans, including: 1) subjecting the subject to the therapeutic agent or
treatment; 2) introducing a caspase affinity labeling agent into the subject; 3)
drawing blood and preparing peripheral blood monocytes (PBMCs); and 4)
detecting the presence or abundance of the affinity labeling agent in the cells;
20 wherein the presence or abundance of apoptosis correlates with treatment
efficacy.

Certain embodiments of the invention provide a method for determining
if one or more compounds, *e.g.*, within a chemical library, modulate caspase
activity in a mammal including,
25 contacting the mammal, including humans, with one or more compounds,
and
contacting the subject as with a caspase affinity labeling agent; and
determining the level of the affinity labeling agent in the subject, and
comparing the level of affinity labeling agent in the subject with a control
30 subject not exposed to the compound to determine whether the compound
modulated the caspase activity.

Certain embodiments of the invention provide a method for determining if one or more compounds, *e.g.*, within a chemical library, induces apoptosis in mammals, including humans, including,

5 contacting the subject with one or more compounds, and
 contacting the subject with an apoptosis affinity labeling agent; and
 determining the level of the affinity labeling agent in the subject, and
 comparing the level of affinity labeling agent in the subject with a control
subject not exposed to the compound to determine whether the compound
induces apoptosis.

10 Certain embodiments of the invention provide a method for determining
if one or more compounds, *e.g.*, within a chemical library, reduces apoptosis in
mammals, including humans, including,
 contacting the subject with one or more compounds, and
 contacting the subject with an apoptosis affinity labeling agent; and
15 determining the level of the affinity labeling agent in the subject, and
 comparing the level of affinity labeling agent in the subject with a control
subject not exposed to the compound to determine whether the compound
reduces apoptosis.

 Certain embodiments of the invention provide a method for longitudinal
20 determination if one or more compounds, *e.g.*, within a chemical library,
modulate caspase activity in a mammal including,

 contacting the subject as with a caspase affinity labeling agent before
contacting the subject with one or more compounds; and
 determining the level of the affinity labeling agent in the subject, and
25 contacting the mammal, including humans, with one or more compounds,
then
 contacting the subject as with a caspase affinity labeling agent; and
 determining the level of the affinity labeling agent in the subject, and
 comparing the level of affinity labeling agent in the subject before
30 exposure to the compound to determine whether the compound modulated the
caspase activity.

Certain embodiments of the invention provide a method for determining if one or more compounds, *e.g.*, within a chemical library, induces apoptosis in mammals, including humans, including,

- contacting the subject as with a caspase affinity labeling agent before
- 5 contacting the subject with one or more compounds; and
- determining the level of the affinity labeling agent in the subject, and
- contacting the mammal, including humans, with one or more compounds,
- then
- contacting the subject as with a caspase affinity labeling agent; and
- 10 determining the level of the affinity labeling agent in the subject, and
- comparing the level of affinity labeling agent in the subject before
- exposure to the compound to determine whether the compound induces caspase activity.

Certain embodiments of the invention provide a method for determining

15 if one or more compounds, *e.g.*, within a chemical library, reduces apoptosis in mammals, including humans, including,

- contacting the subject as with a caspase affinity labeling agent before
- contacting the subject with one or more compounds; and
- determining the level of the affinity labeling agent in the subject, and
- 20 contacting the mammal, including humans, with one or more compounds,
- then
- contacting the subject as with a caspase affinity labeling agent; and
- determining the level of the affinity labeling agent in the subject, and
- comparing the level of affinity labeling agent in the subject before
- 25 exposure to the compound to determine whether the compound reduces caspase activity.

In certain embodiments of the invention, detection is carried out using NMR, MRI, CT, CAT, PET scans, or scintigraphy; flow cytometer; a laser scanning cytometer; a fluorescence microplate reader; a luminescence microplate

30 reader, a chromogenic microplate reader; a fluorescence microscope; a confocal microscope; a luminescence microscope; a bright-field microscope; whole

animal fluorescence imaging systems (optical imaging systems); or a whole animal luminescence imaging system.

In certain embodiments of the invention, detection is carried out using a Window Chamber inserted into the test subject for direct observation.

5 In certain embodiments of the invention, detection is carried out using a fluorescence microscope; a confocal microscope; a bright-field microscope, or luminescence microscope.

In certain embodiments of the invention, detection is carried out and/or confirmed by removing a sample from the test subject such as by extraction,
10 biopsy, venipuncture, dissection, or other suitable methods and detection by flow cytometer; a laser scanning cytometer; a fluorescence microplate reader; a chromogenic microplate reader; a fluorescence microscope; a confocal microscope; a bright-field microscope; a luminescence microplate reader; or a luminescence microscope.

15 Certain embodiments of the invention provide a kit such as an assay kit including packaging materials including 1) one or more caspase affinity labeling agents; 2) 10X injection buffer; and 3) instructions for using the compound to determine the level of apoptosis *in vivo*.

The invention has been described with reference to various specific and
20 preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit, and scope, of the invention.

Example 1

Using a mouse window chamber model, SCK mammary tumors were
25 evaluated for levels of apoptosis induction in tumors following irradiation treatment with arsenic trioxide (ATO) and heat. A window chamber, used for viewing tumors, was placed over a fold of skin in A/J mice. 2×10^5 SCK mammary carcinoma cells were injected into the skin fold. The tumors were allowed to grow for 7 to 9 days. In the test mice, tumors were irradiated with 20
30 Gy, 8 mg/kg ATO and 2 hours later the tumors were heated at 41.5°C for 60 minutes. Control mice were not treated with irradiation or heat and a placebo was injected instead of ATO.

After allowing the tumors to grow 7 days, the test mice were first irradiated with 20 Gy. This was immediately followed by an intraperitoneal (I.P.) injection of ATO. The ATO was dissolved in water and injected in a solution of PBS with 5% w/v of dextrose at a concentration of 8 mg/kg. After 2
5 hours the tumors were heated at 41.5°C for 60 minutes. Control mice received an I.P. injection of PBS with 5% dextrose.

Prior to treatment, tumors were examined in control mice and designated test mice. Low magnification bright-field image composite photographs of SCK mammary tumors taken through a window chamber reveal a low level of
10 hemorrhaging in all tumors (Figure 1).

Three hours post treatment, low magnification bright-field image composite photographs show continued low levels of hemorrhaging in control mice and high levels of hemorrhaging in test mice (Figure 2) demonstrating the efficacy of the treatment. After 24 hours the control mice had the same levels of
15 hemorrhaging and the levels of hemorrhaging continued to increase in the test mice that received the treatment (Figure 3).

Apoptosis was detected by injecting the *in vivo* apoptosis detection reagent FAM-VAD-FMK into the tail vein of control and test mice. A processed vial of reagent containing 52 µg of FAM-VAD-FMK was dissolved into 50 µL
20 of DMSO. This solution was diluted by adding 200 µL of sterile 1X injection buffer. Of the diluted reagent, 40 µL (8.3 µg) was injected I.V. through the tail vein and allowed to circulate in the mouse for 30 minutes. The level of apoptosis in each tumor was viewed directly through the window chambers at high magnification with an excitation wavelength of 488 nm and using a FITC
25 filter.

Control mice were given FAM-VAD-FMK injections following placebo injections. These mice showed a low level of apoptosis, as would be expected in a fast growing tumor (Figure 4). Test mice that received ATO treatment were given FAM-VAD-FMK injections at 3 hours and 24 hours after ATO treatment,
30 and apoptosis was evaluated as before. After 3 hours, there was a substantial increase in the level of apoptosis, as seen in Figure 5. The level of apoptosis was even higher after 24 hours (Figure 6).

In another set of mice, the tumors were allowed to grow for 7 days. Control mice received placebo injections as before and test mice received ATO injections as before. After 24 hours all mice received I.V. injections of FAM-VAD-FMK as before. The *in vivo* apoptosis detection and imaging reagent was
5 allowed to circulate through each mouse for 30 minutes. After imaging tumor-associated apoptosis *in vivo*, tumor tissue was collected from the window chamber by scraping the tumor out of the chamber into a trypsin solution, stirring for 30 minutes with 10 µg/ml DNase and 5 µg/ml collagenase, filtering the suspension using a 70 µM cell strainer. Flow cytometry was then performed
10 using a FACS Caliber flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) for the analysis of apoptosis in the cell population obtained from the window chamber. All data was acquired with an event acquisition set for 10,000 events. Data was analyzed using CellQuest Pro cytometer software. The results indicated a two-fold increase in apoptosis with
15 the control mouse having 18% apoptotic cells and the test mouse having 39% apoptotic cells (Figure 7).

Example 2

A longitudinal study was performed in nu/nu mice to assess the efficacy of arsenic trioxide (ATO) on FSaII murine fibrosarcoma tumors before and after
20 ATO treatment through a window chamber.

Dorsal skin flap chambers (DSFC) were surgically implanted into the backs of nu/nu mice as follows. Entire implantation procedure was conducted within a laminar flow hood to preserve a sterile field. Skin was washed twice with betadine and allowed to dry. One sterilized chamber piece containing a
25 round window (9 mm) was placed on one side of the dorsal skin fold. Holes for three connecting screws were made through the skin fold connecting with another chamber piece with a similar window. The assembly was then tightened together with screws and nuts through the chamber piece spacers. A circle of cutaneous tissue and fascia was carefully cut away from the skin fold inside
30 the DSFC window, exposing the blood vessels of the subcutaneous tissue adjacent to the striated muscles of the opposing skin fold. Antibiotic ointment (bacitracin zinc/polymyxin B sulfate/neomycin sulfate) was used on the edges of

the wound inside the DSFC window. A glass window was placed in the chamber to cover the exposed tissue and secured with a snap C-ring. The surgical wound procedure was then repeated in the opposite DSFC window so that a very thin, transparent layer of fascia and vessels remained visible through both windows
5 (created to facilitate microscope light transmission through the tissue). Finally, the chamber was sutured to the skin.

After surgery, the animals were housed in barrier cages inside a humidified incubator maintained at 32°C with unrestricted access to food and water. To minimize risk of infection, all mice were given amoxicillin in their
10 drinking water (250 mg per 5 mL water) after DSFC implantation and throughout the experiment.

Tumor cells were implanted into one side of the chamber by temporary removal of the C-ring and glass cover slip. 1×10^5 FSaII murine fibrosarcoma tumor cells in 10 μ L Matrigel (BD Biosciences, Bedford, MA) were added on
15 top of the fascia and the window closed back over the tissue as described above. Cells were placed within the chamber 24 hrs post surgery. Mice were checked daily for general health as well as for vessel presence, tumor progression and chamber-skin interaction. Images were taken of the tumor by removing the C-ring and glass cover slip and attaching the chamber of the anesthetized mouse to
20 a modified microscope stage.

Anti-vascular agent and administration: Arsenic trioxide (ATO) was originally obtained from Cell Therapeutics, Inc. (Seattle, WA) under the brand name, Trisenox, at a concentration of 1.0 mg/mL. Mice to be injected were weighed and ATO was injected IP at a dose of 8.0 mg ATO per kilogram of
25 mouse body weight.

In vivo apoptosis detection reagent carboxyfluorescein-valanyl-alanyl-aspartyl(O-methyl)-fluoromethyl ketone (FAM-VAD-FMK), preparation and administration: A processed vial of reagent containing 52 μ g of FAM-VAD-FMK was dissolved into 50 μ L of DMSO. This solution was diluted by adding
30 250 μ L of sterile 1X injection buffer. Of the diluted reagent, 70 μ L (12.0 μ g) was injected IV through the tail vein and allowed to circulate in the mouse for 45 minutes. The level of apoptosis in each tumor was viewed directly through the

window chambers at 10X magnification with an excitation wavelength of 488 nm and using a FITC filter.

Results: The tumors were allowed to grow for 9 days. FAM-VAD-FMK was injected and allowed to circulate for 45 minutes. The DFSC C-ring and glass cover were removed and the mouse was connected to the microscope. A 10X photograph was taken using a 488 nm excitation with a FITC filter (Figure 8). This photograph shows a lack of apoptosis occurring in the tumor. The same mouse was then injected with ATO as described above. The ATO was allowed to work for 3 hours before the mouse was injected a second time with FAM-VAD-FMK. After 45 minutes the mouse was again connected to the microscope and the same tumor was photographed (Figure 9). This photograph shows a significant level of apoptosis in the mouse tumor.

Final conclusion: The IV injection of a *in vivo* apoptosis detection reagent (FAM-VAD-FMK) works for the *in vivo* detection of apoptosis in living tissue in an animal. It can also be used to perform longitudinal studies in the same animal with no side-effects from the reagent. This method represents a new and valuable method to reliably demonstrate anti-cancer drug activity in living normal or malignant tissue.

Example 3

The objective of this project was to develop an *in vivo* animal model for the detection of morphine-mediated apoptosis. Drugs of abuse, including morphine, have been shown to induce apoptosis in cultured cells. Apoptosis is initiated through a variety of complex pathways that lead to the activation of cysteine proteases known as caspases. The detection of apoptosis is important for the understanding of the adverse effects of drugs, *e.g.*, drugs of abuse, *e.g.*, morphine. Although there are techniques for the detection of apoptosis, those techniques are generally limited to removing the cells from their natural environment. This mechanical processing is known to induce apoptosis, potentially resulting in exaggerated positive results. The objective of this study was to develop an *in vivo* model for the detection of apoptosis induced by morphine.

To test the effects of morphine, the mice were divided into 4 groups,

dependent on the treatment. One group received a placebo, a second group received LPS to stimulate cells, a third group received morphine, and a fourth group received morphine and LPS. After the mice were given their respective treatments, they received IV injections of FAM-VAD-FMK. A processed vial of
5 reagent containing 52 μg of FAM-VAD-FMK was dissolved into 50 μL of DMSO. This solution was diluted by adding 200 μL of sterile 1X injection buffer. Of the diluted reagent, 40 μL (8.3 μg) was injected IV through the tail vein and allowed to circulate in the mouse for 45 minutes.

Spleen, Liver, Thymus, and CNS tissues were isolated from different
10 treatment groups, frozen and mounted on glass slides. The various cell types were adjusted to 1×10^6 cells/mL and cytopun on a glass slide for analysis by fluorescence microscopy. Sections were photographed and analyzed by MetaMorph analysis program for apoptosis positive cell counts. Counts were analyzed by statistics software. Morphine statistically enhanced LPS induced
15 apoptosis in the Spleen and Thymus ($p=0.012$ and 0.0018 respectively). An increase in apoptosis was detected in the liver and central nervous system, however, they were not statistically significant. Morphine alone did not induce apoptosis. Quantitative results are summarized in Table 1.

Conclusion: In contrast to previously published reports on morphine
20 mediating *in vitro* apoptosis of immune cells, this study demonstrates that morphine alone did not significantly induce apoptosis. However, morphine did enhanced apoptosis of LPS activated cells detected in spleen, bone marrow and liver tissues.

Table 1 is a summary of quantitative results of apoptosis induced by LPS,
25 morphine, and morphine + LPS in the spleen, thymus, liver and CNS. *In vivo* apoptosis detection was made by intravenous injection of 8.3 μg of FAM-VAD-FMK with a 45 minute incubation time. Spleen, Liver, Thymus, and CNS tissues were isolated from different treatment groups, frozen and mounted on glass slides. Sections were photographed and analyzed by MetaMorph analysis
30 program for apoptosis positive cell counts. Counts were analyzed by statistics software.

Table 1

Tissues	Treatments	Animals	Counts
Spleen	Placebo	1/4	26
	Morphine	2/4	39 ± 12
	LPS	3/4	48 ± 6
	Morphine + LPS	4/4	117 ± 23*
Liver	Placebo	1/4	11
	Morphine	3/4	12 ± 3
	LPS	3/4	17 ± 5
	Morphine + LPS	4/4	78 ± 16
Thymus	Placebo	0/4	0
	Morphine	2/4	12 ± 6
	LPS	2/4	21 ± 7
	Morphine + LPS	4/4	103 ± 13*
CNS	Placebo	0/4	0
	Morphine	0/4	0
	LPS	1/4	6.5
	Morphine + LPS	2/4	22.5 ± 28

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All publications and patent documents cited herein are incorporated by
reference herein, as though individually incorporated by reference.

- 15 The invention has been described with references to various specific and
preferred embodiments and techniques. However, it should be understood that
many variations and modifications may be made while remaining within the
spirit and scope of the invention.

Claims

What is claimed is:

1. A method for *in vivo* determination of the apoptotic state of one or more cells in an animal, comprising detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the presence or abundance of the caspase affinity labeling agent correlates with the apoptotic state of the cells.
2. A method for *in vivo* determination of whether a therapeutic agent modulates apoptosis in one or more cells in an animal, comprising detecting the presence of at least one caspase affinity labeling agent in the cells of an animal that has been previously treated with the therapeutic agent and into which at least one caspase affinity labeling agent has been introduced, wherein the presence of the caspase affinity labeling agent correlates with the ability of the therapeutic agent to modulate apoptosis.
3. The method of claim 2, wherein the method determines whether the therapeutic agent increases or decreases apoptosis.
4. A method for *in vivo* determination of whether a radiation treatment modulates apoptosis in one or more cells in an animal, comprising detecting the presence of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the animal has been previously treated with radiation, wherein the presence of the caspase affinity labeling agent correlates with the ability of the radiation to modulates apoptosis.
5. The method of claim 4, wherein the method determines whether the radiation treatment increases or decreases apoptosis.

6. An *in vivo* diagnostic method for determining the presence or absence of a disease characterized by the presence of apoptosis comprising detecting the presence of at least one caspase affinity labeling agent in the cells of the animal into which at least one caspase affinity labeling agent has been introduced, wherein the presence or absence of at least one caspase affinity labeling agent correlates with the presence or absence of the disease.
7. The method of claim 6 wherein the disease is a disease of the eye, a neurodegenerative disease, or a cardiac disease.
8. The method of claim 7 wherein the disease is glaucoma, macular degeneration, proliferative retinopathy, neuropathy, Alzheimer's disease, multiple sclerosis, or Huntington's disease.
9. An *in vivo* method for evaluating the sensitivity of a disease to at least one therapeutic agent or treatment, comprising detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the animal has previously received the therapeutic agent or treatment, wherein the presence or abundance of the caspase affinity labeling agent correlates with the sensitivity of the disease to at least one therapeutic agent or treatment.
10. A method for the monitoring of cancer treatment in an animal, comprising detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of the animal into which at least one caspase affinity labeling agent has been introduced that has also received a therapeutic agent or treatment, wherein the presence or abundance of at least one caspase affinity labeling agent correlates with the efficacy of the therapeutic agent or treatment.
11. A method for the monitoring of leukemia treatment in an animal, comprising detecting the presence or abundance of at least one caspase affinity

labeling agent in the cells of the animal into which at least one caspase affinity labeling agent has been introduced that has also received a therapeutic agent or treatment, wherein the presence or abundance of at least one caspase affinity labeling agent correlates with the efficacy of the therapeutic agent or treatment.

12. A method for the monitoring of blood and bone marrow disease treatment in an animal comprising detecting the presence or abundance of at least one caspase affinity labeling agent in the cells of an animal into which at least one caspase affinity labeling agent has been introduced that has also received a therapeutic agent or treatment, wherein the presence or abundance of at least one caspase affinity labeling agent correlates with the efficacy of the therapeutic agent or treatment.

13. A method for determining if one or more compounds modulate caspase activity in an animal comprising determining the level of at least one caspase affinity labeling agent in cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the animal has also been contacted with one or more compounds, and determining whether the one or more compounds modulates the caspase activity.

14. A method for determining if one or more compounds modulates apoptosis in an animal comprising determining the level of at least one caspase affinity labeling agent in cells of an animal into which at least one caspase affinity labeling agent has been introduced, wherein the animal has been contacted with one or more compounds, and determining whether the one or more compounds modulates apoptosis.

15. The method of claim 13 or 14, wherein the determination step comprises comparing the level of affinity labeling agent in the animal with a control animal not exposed to the compound.

16. The method of claim 13 or 14, wherein the determination step is a longitudinal study that comprises comparing the level of affinity labeling agent in the animal before exposure to the compound and after the animal has been exposed to the compound.
17. A method of any one of claims 1-16 wherein detection is carried out using NMR, MRI, CT, CAT, PET scan, scintigraphy, a flow cytometer, a laser scanning cytometer, a fluorescence microplate reader, a luminescence microplate reader, a chromogenic microplate reader, a fluorescence microscope, a confocal microscope, a luminescence microscope, a bright-field microscope, a whole animal fluorescence imaging system, a whole animal luminescence imaging system, or a combination thereof.
18. A method of any one of claims 1-17 wherein detection is carried out using a window chamber that has been inserted into the animal.
19. A method of claim 18 wherein detection is carried out using a fluorescence microscope, a confocal microscope, a bright-field microscope, or a luminescence microscope.
20. A method of any one of claims 1-19 wherein detection is carried out or confirmed by removing a sample from the animal such as by extraction, biopsy, venipuncture, dissection, or other suitable methods and detection is carried out on a sample that has been removed from the animal.
21. The method of claim 20, wherein the detection is via flow cytometer; a laser scanning cytometer; a fluorescence microplate reader; a chromogenic microplate reader; a fluorescence microscope; a confocal microscope; a bright-field microscope; a luminescence microplate reader; or a luminescence microscope.

22. The method of any one of claims 1-21, wherein the presence or abundance of the affinity labeling agent is detected in the bone marrow, thymus, lymph nodes, spleen or circulating blood of the animal.
23. The method of any one of claims 1-21, wherein the presence or abundance of the affinity labeling agent is detected in peripheral blood monocytes.
24. The method of any one of claims 1-23, wherein the cells are comprised in tissues, organs or tumors of the animal.
25. The method of any one of claims 1-24, wherein the caspase affinity labeling agent is introduced into the animal by intravenous, intravascular, intraperitoneal, intravitreal, intraocular, intracranial, intrapleural, intrathoracic, intramuscular, intrapulmonary, injection, perfusion, or lavage administration.
26. The method of any one of claims 1-25, wherein the animal is a mammal.
27. The method of claim 26, wherein the mammal is a human.
28. The method of any one of claims 1-27, wherein the caspase affinity labeling agent is a compound of formula I:



wherein:

- L_1 is a detectable group;
- A_1 is a direct bond or a linker;
- X_1 is absent, an amino acid, or a peptide; and
- R_1' comprises an aspartic acid side-chain, an ester of aspartic acid, an aza-peptide epoxide modification of the aspartic acid, an aza-peptide Michael acceptor, an aldehyde modification of the aspartic terminal carboxyl group, a chloromethyl ketone group, or an acyloxy reactive group.

29. The method of claim 28, wherein the detectable group comprises a Lanthanide series element.
30. The method of claim 28, wherein the detectable group comprises a positron emitter.
31. The method of claim 28, wherein the detectable group comprises a fluorescent label.
32. The method of claim 28, wherein the detectable group comprises a radioisotope.
33. The method of claim 28, wherein the detectable group comprises Gd, Tb, Eu, Ce, Pr, Nd, Pm, Sm, Dy, Ho, Er, Tm, Yb, Lu, Fe, Mn, Re, and Tc, I Ba, ^{11}C , ^{13}N , ^{15}O , ^{64}Cu , a fluorescein derivative, a sulforhodamine derivative, a Cy dye derivative, a BODIPY derivative, a coumarin derivative, a Quantum Dot, ^3H , ^{14}C , or ^{35}S .
34. The method of claim 28, wherein X_1 is V, VA, YVA, DEV, LEE, LEH, VDVA, IET, WHE, AEV, A, V, or E.
35. The method of claim 28, wherein R_1' is $\text{CH}_2\text{-COOH}$, or $\text{-CH}_2\text{CO}_2\text{R}$, wherein R is $\text{C}_1\text{-C}_6$ alkyl or benzyl, CH_3 , C_2H_5 or $\text{CH}_2\text{C}_6\text{H}_5$.
36. The method of any one of claims 1-27, wherein the caspase affinity labeling agent is carboxyfluorescein-valanyl-alanyl-aspartyl(O-methyl)-fluoromethyl ketone.
37. The method of any one of claims 1-36, wherein the caspase affinity labeling agent is cell permeant.

38. The method of any one of claims 1-37, wherein the caspase affinity labeling agent inhibits active caspases by binding covalently to the active catalytic site and is retained within the cell.
39. An assay kit comprising packaging materials, one or more caspase affinity labeling agents, injection buffer, and instructions for using the caspase affinity labeling agents to determine the level of apoptosis *in vivo*.
40. The kit of claim 39, wherein the one or more caspase affinity labeling agents includes a compound as described in any one of claims 28-38.

Figure 1. SCK mammary tumor in A/J mice, Brightfield Image
Time: 0 hours

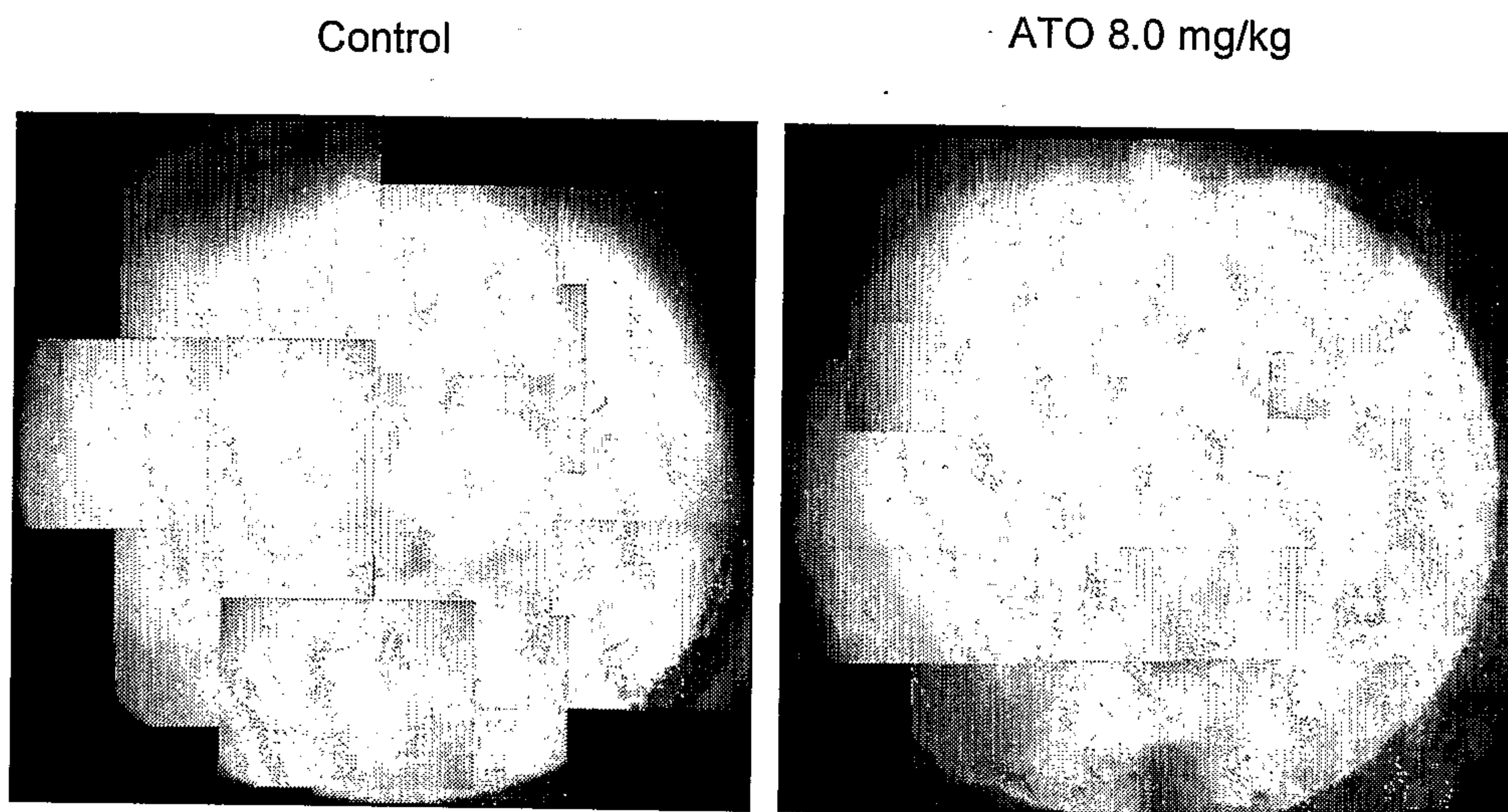


Figure 2. SCK mammary tumor in A/J mice, Brightfield Image
Time: 3 hours

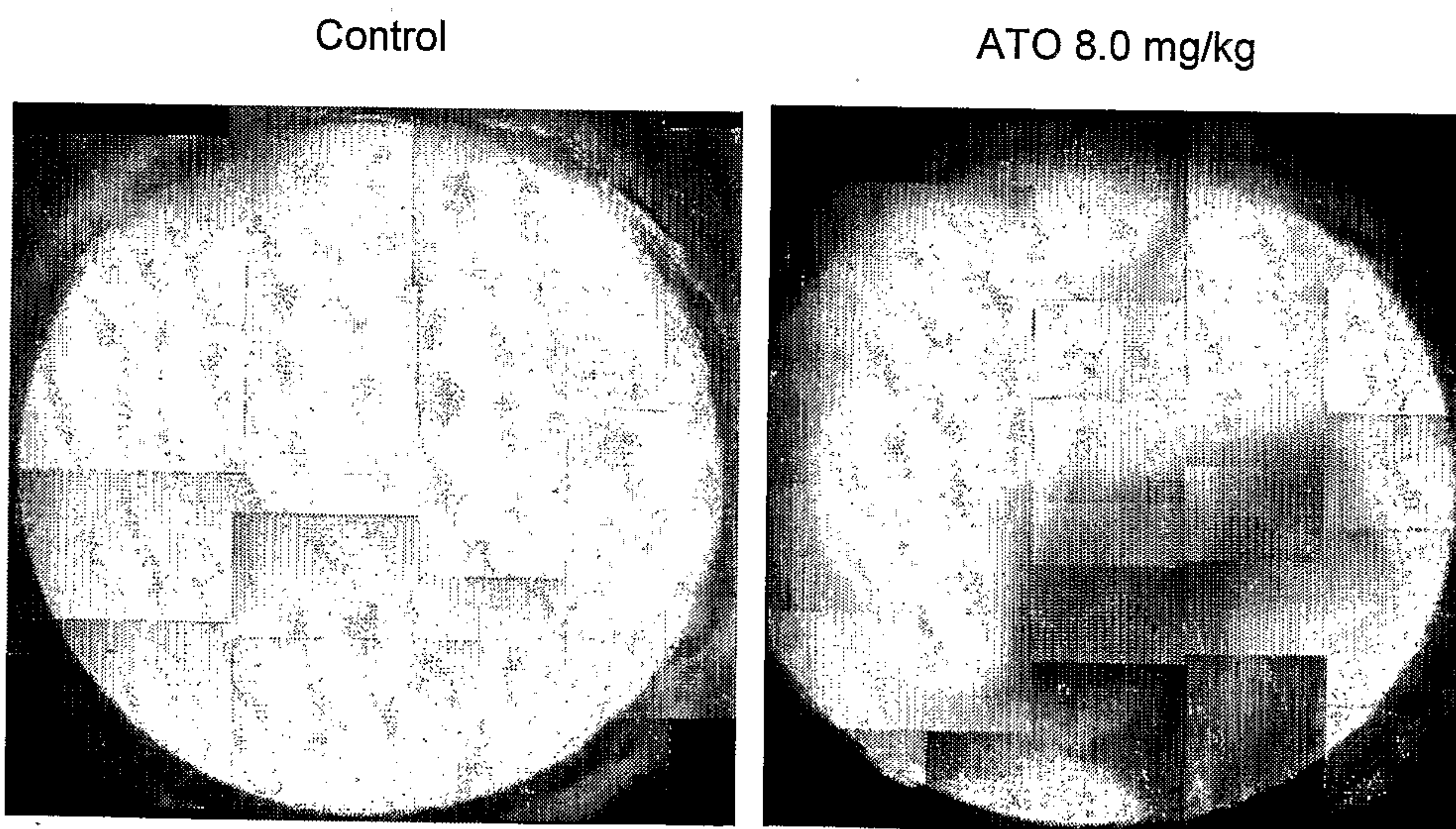


Figure 3. SCK mammary tumor in A/J mice, Brightfield Image,
Time: 24 hours

Control

ATO 8.0 mg/kg

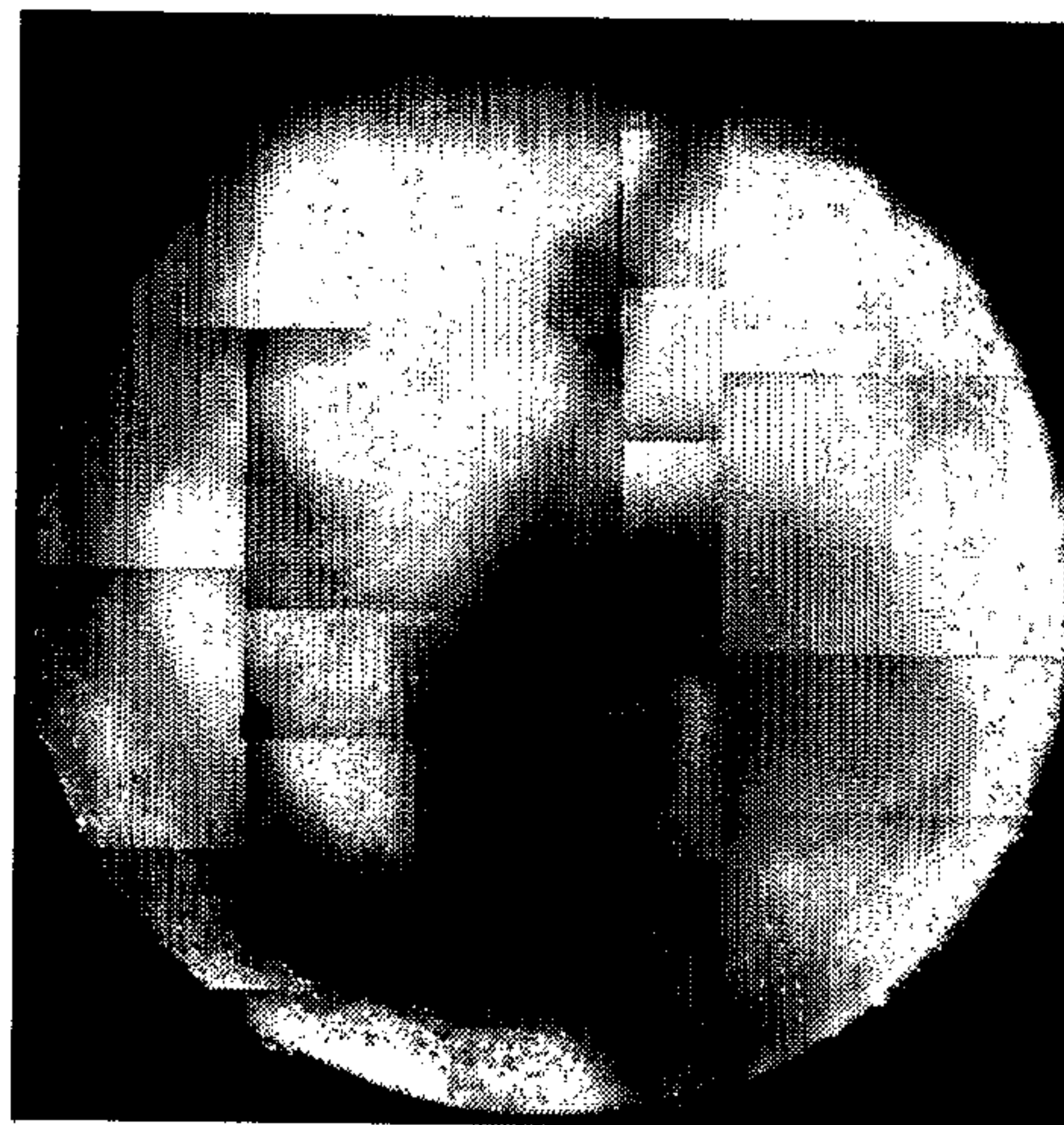
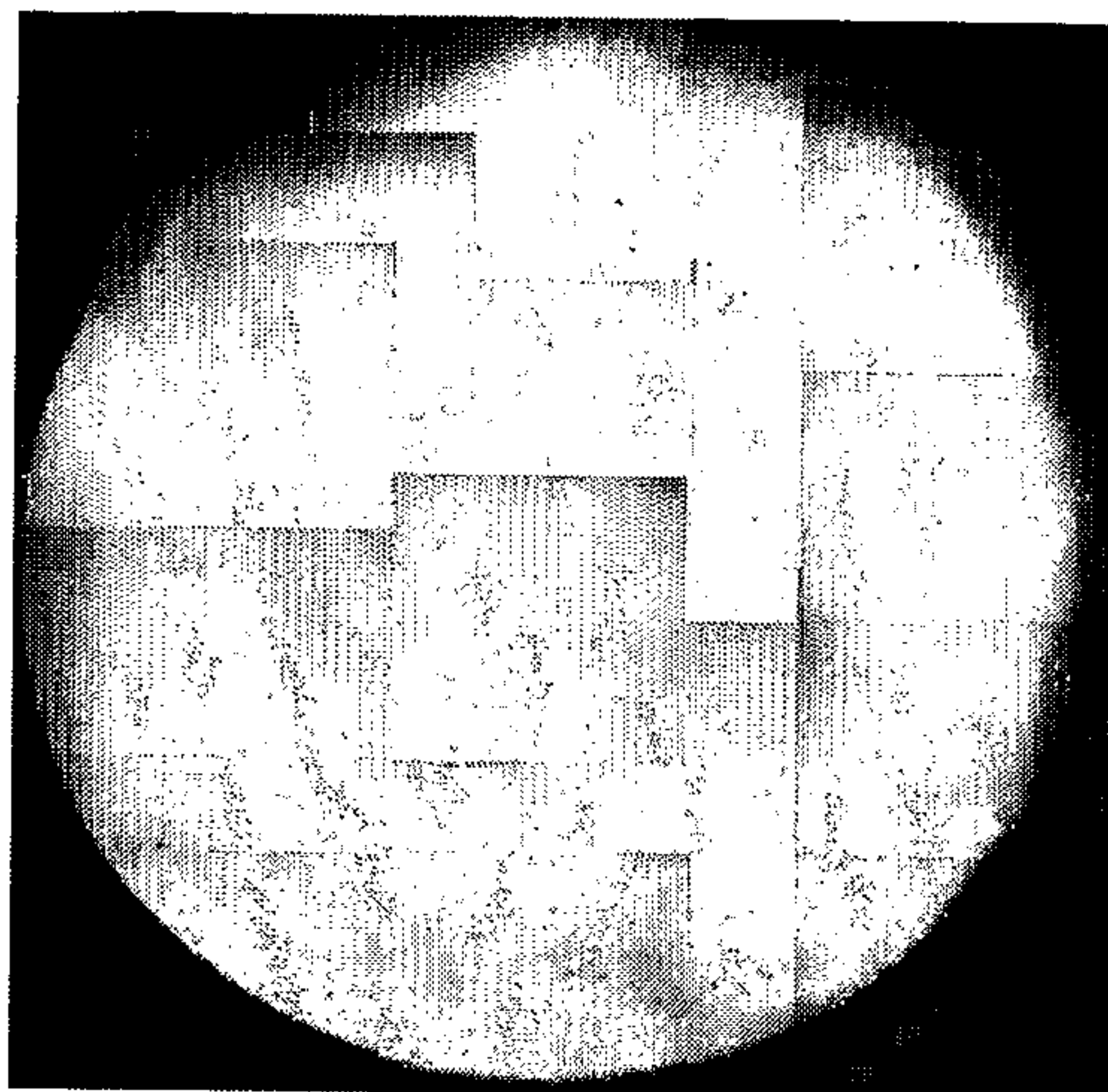


Figure 4. SCK mammary tumor in A/J mice, injected intravenously
with 8.0 μ g of FAM-VAD-FMK, 0 Hours

Post Treatment Time: 0 Hours, ATO 8.0 mg/kg

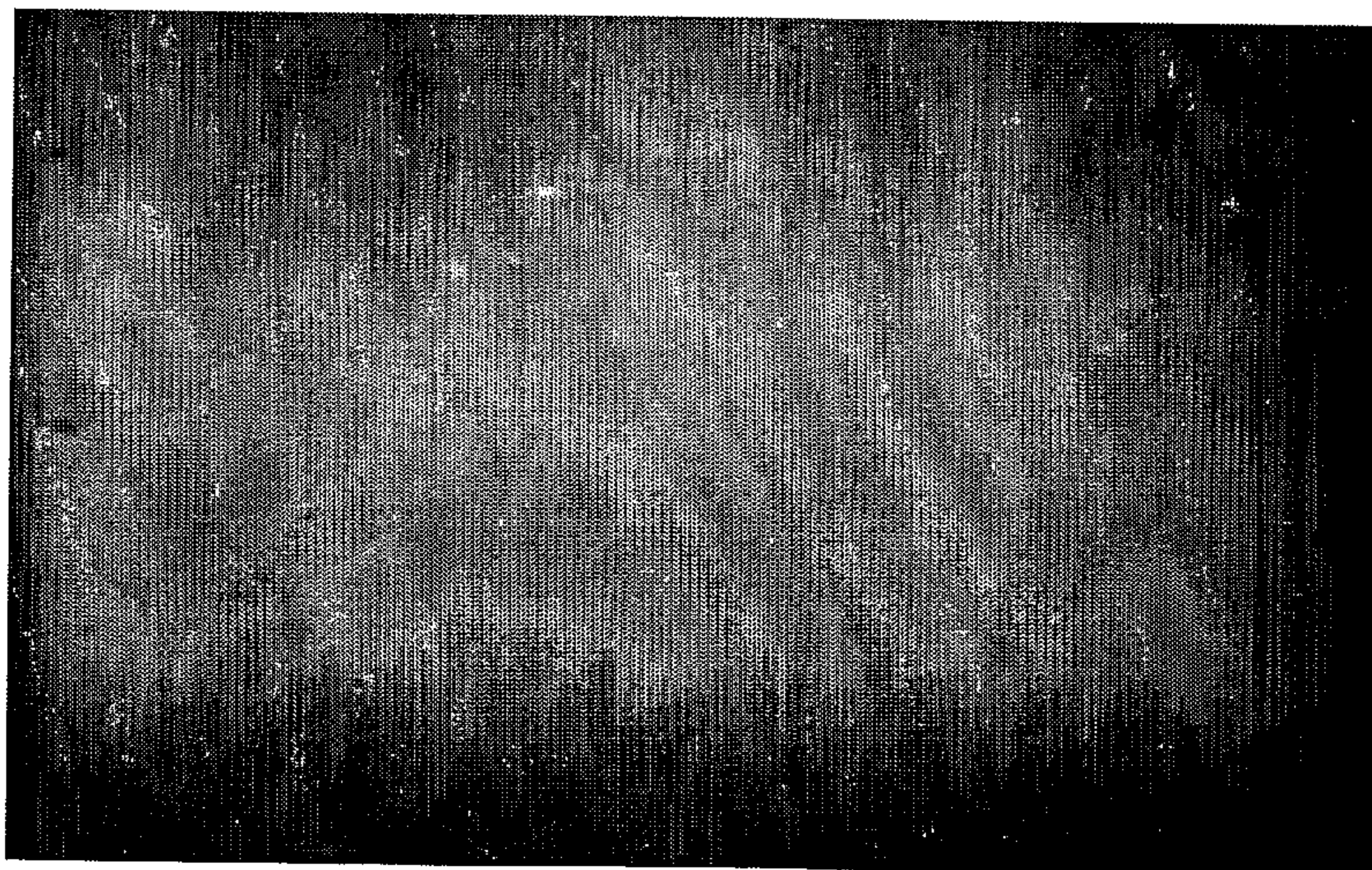


Figure 5. SCK mammary tumor in A/J mice, injected intravenously with 8.0 μ g of FAM-VAD-FMK, 3 Hours

Post Treatment Time: 3 Hours, ATO 8.0 mg/kg

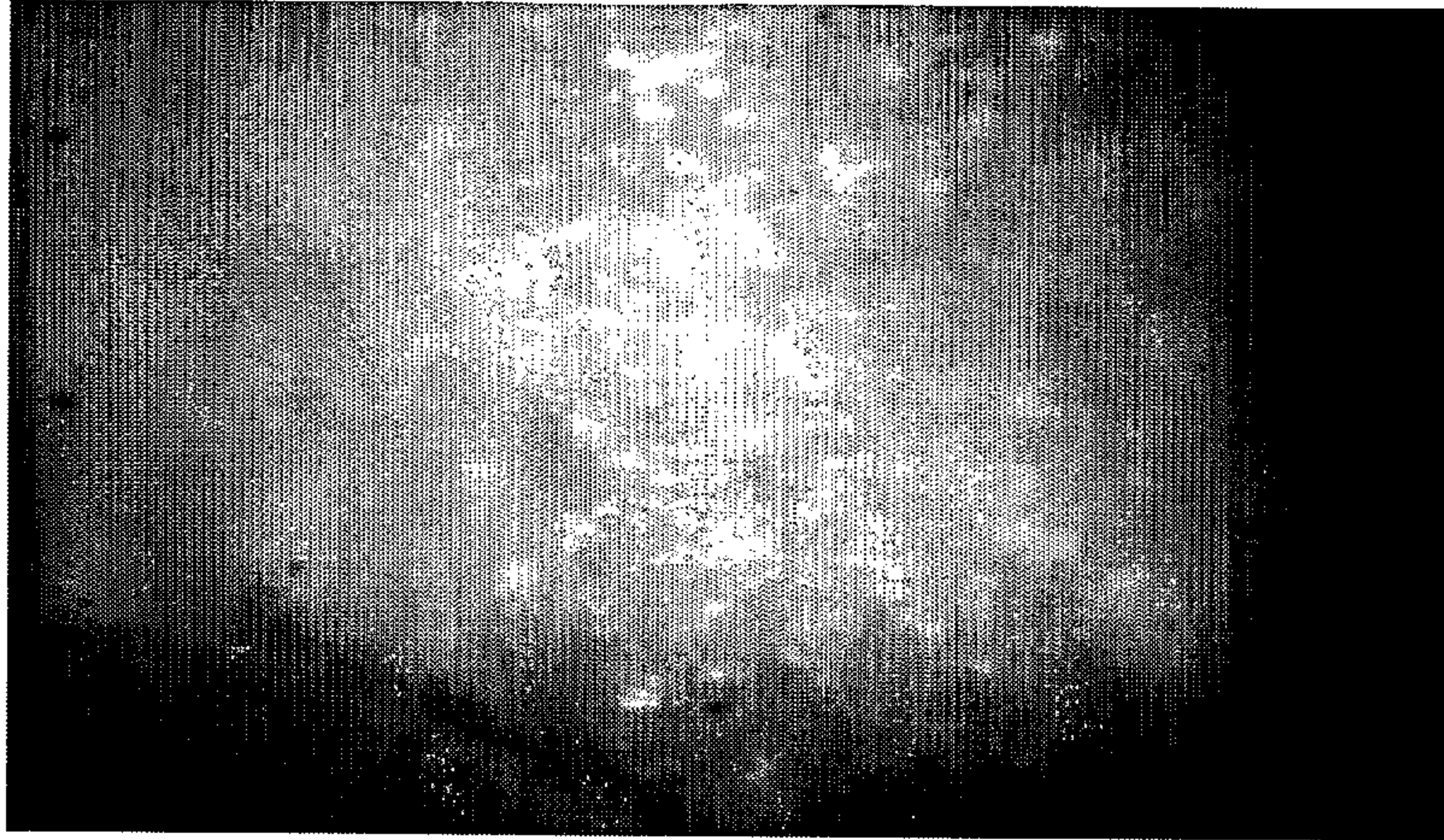


Figure 6. SCK mammary tumor in A/J mice, injected intravenously with 8.0 μ g of FAM-VAD-FMK, 24 Hours

Post Treatment Time: 24 Hours, ATO 8.0 mg/kg

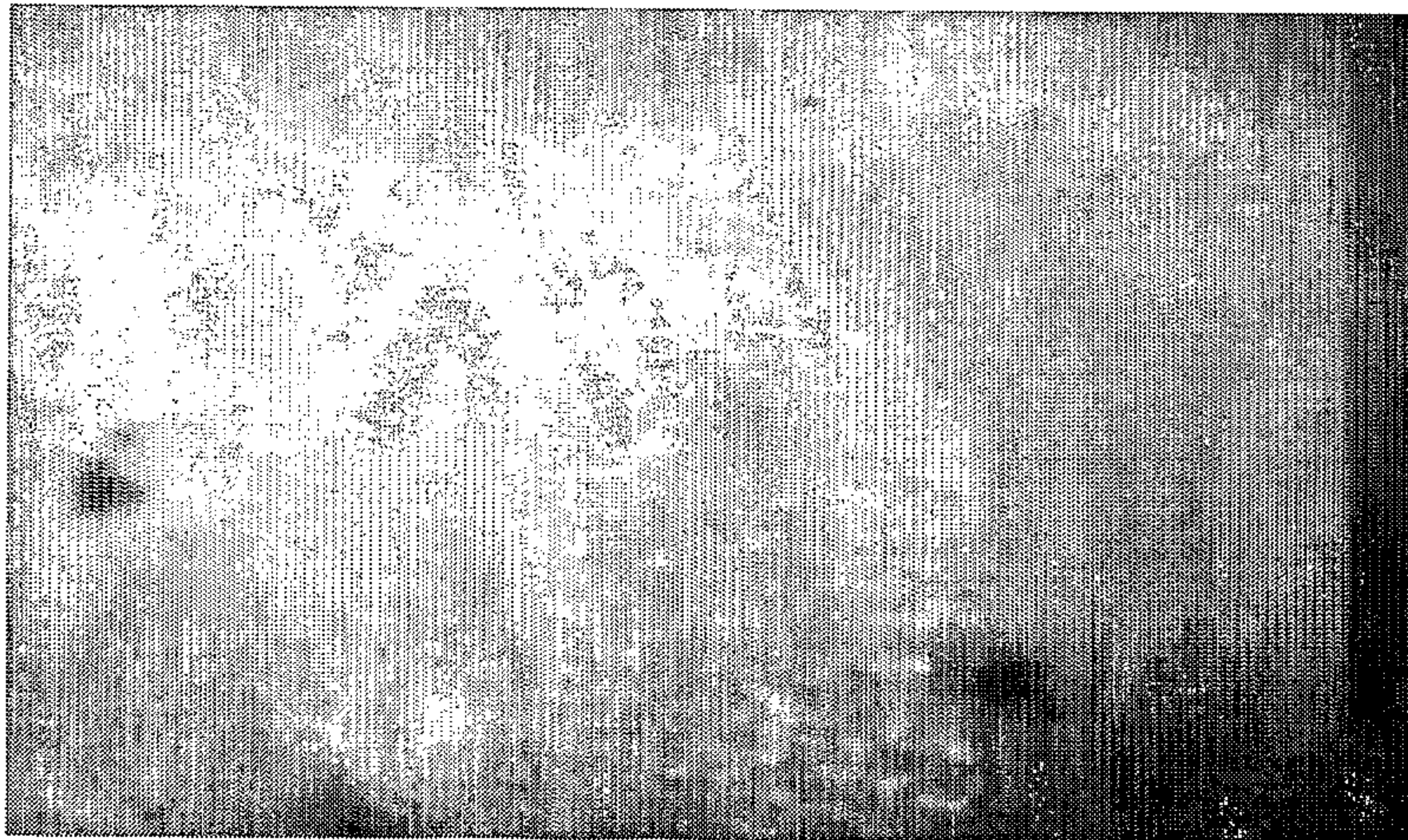


Figure 7. Confirmation by Flow Cytometry using excised tumor cells

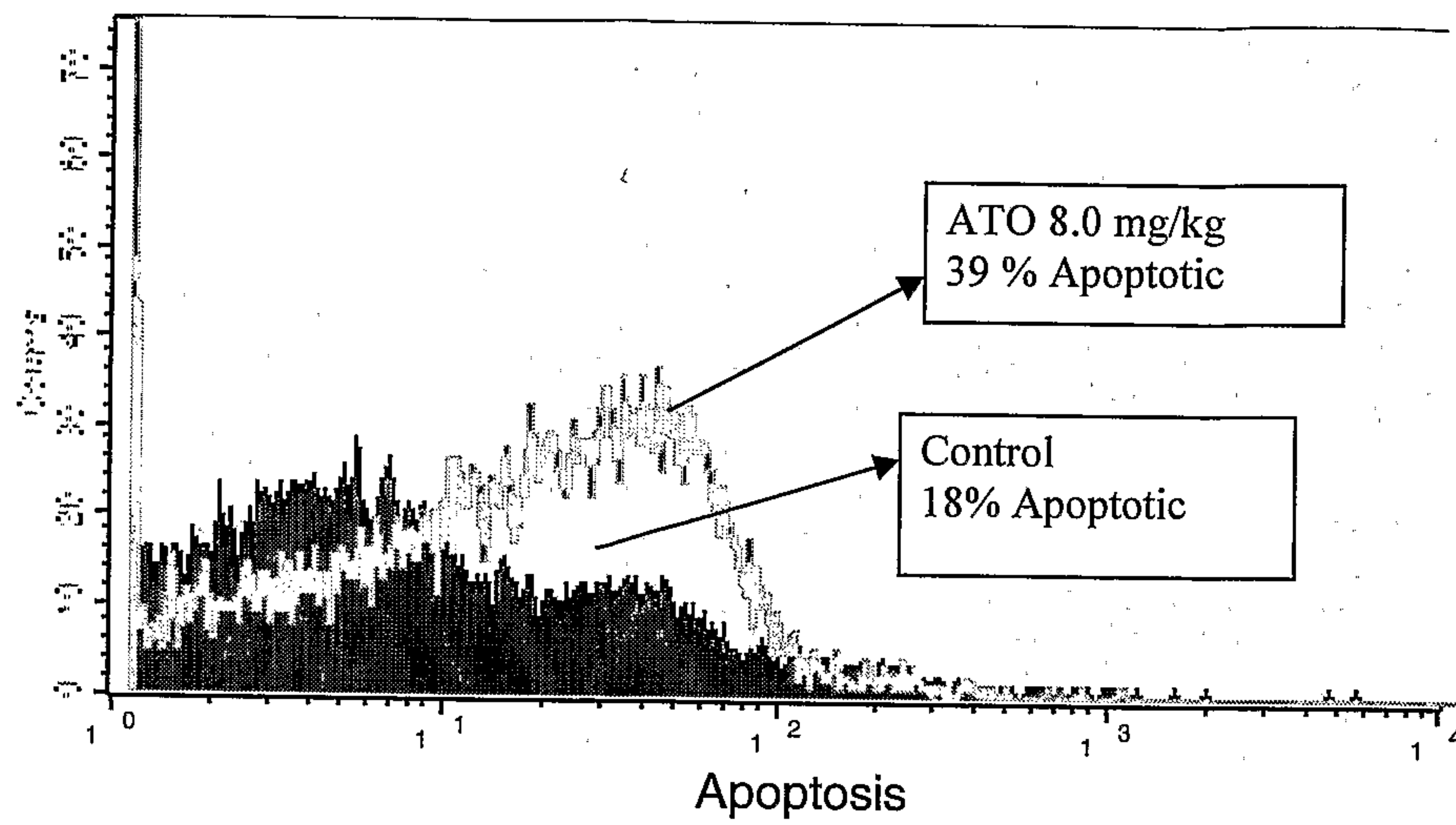


Figure 8. Longitudinal Study: Level of apoptosis in a tumor before treatment with ATO.

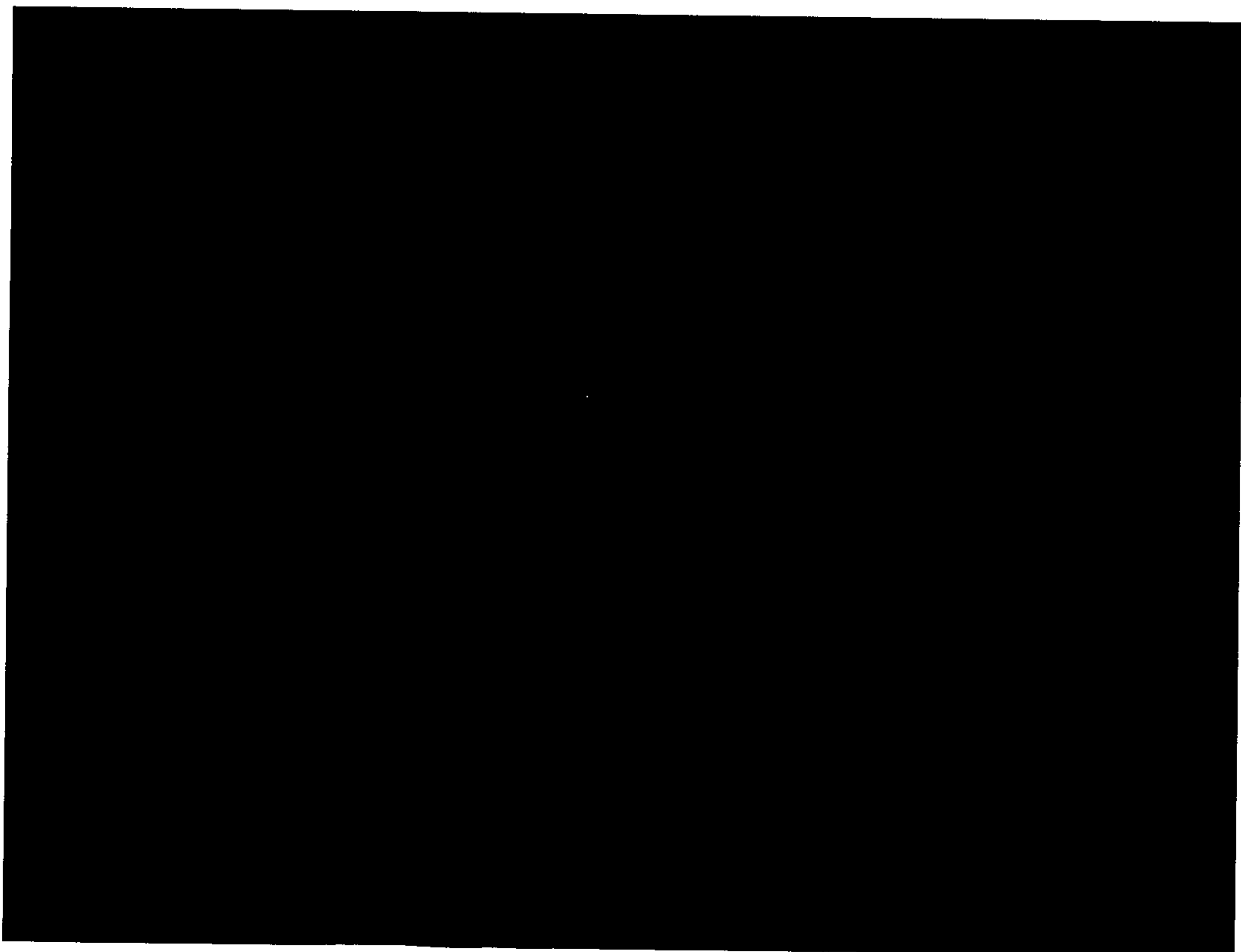
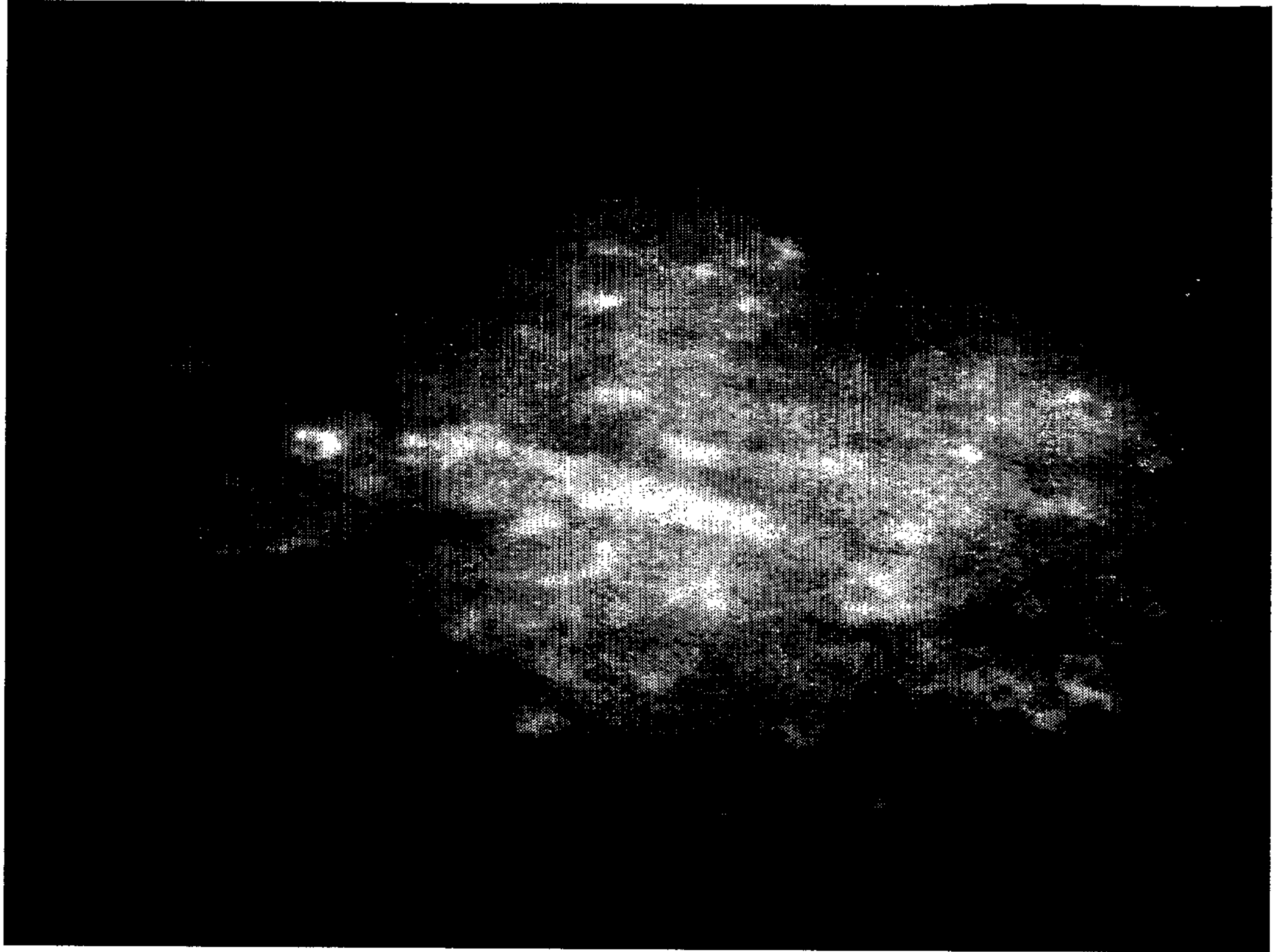


Figure 9. Longitudinal Study: Level of apoptosis in a tumor after 3 hours of treatment with ATO.



SCK mammary tumor in A/J mice, injected intravenously with
8.0 μg of FAM-VAD-FMK, 3 Hours

Post Treatment Time: 3 Hours, ATO 8.0 mg/kg

