Fluorescence of Chromatin DNA Induced by Antitumoral Naphthalimides

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Treatment of chicken blood smears and semithin sections from Epon-embedded mouse tissues with aqueous solutions of the 3-aminonaphthalimides FA-142, FA-2043, and FA-2143 induced a strong green-yellow fluorescence of chromatin under violet or violet-blue excitation. Chromatin emission was abolished by previous DNase or hot TCA treatment. The use of 3-methoxy (FA-655) and 3-nitro derivatives (M-4212 and M-12210) resulted in very weak fluorescence of chromatin. Absorption maxima at 346 and 408 nm and an emission peak at 570 nm were observed for the free compound FA-142. Fluorescence properties open new and interesting applications for some of these antitumoral and DNA-intercalating naphthalimides.

Fluorescence properties of naphthalimides are well known and long used. Some non-ionic naphthalimides are effective fluorescent brighteners for industrial use in fabrics, plastics and laundry products (Allen, 1971; Zollinger, 1987). Anionic (sulfonated) naphthalimides such as brilliant sulfaflavine, brilliant sulfoflavine FF, and Lucifer yellow CH and VS are useful fluorochromes for the cytochemical demonstration of basic and total proteins (Lillie, 1977; Green, 1990). Fluorescent naphthalimides can also form *in situ* by reaction of protein amino groups with 1,8-naphthalic anhydride derivatives (Stockert *et al.*, 1994). However, the use of cationic naphthalimides as fluorescent probes has largely been overlooked in cytochemistry and cell biology.

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Interestingly, strong antitumoral activity (Braña *et al.*, 1980, 1981, 1993; McRipley *et al.*, 1994; Bousquet *et al.*, 1995), intercalative binding to DNA (Waring *et al.*, 1979; Yen *et al.*, 1982), and inhibition of DNA replication and transcription (Braña *et al.*, 1981) have been described for some cationic naphthalimides, although no mention has been made on their use in fluorescence methods. Therefore, the aim of this work is to describe the microscopic fluorescence and emission characteristics of some antitumoral naphthalimides.

Smears of chicken blood were fixed in methanol for 2 min and air-dried. Samples of mouse liver, kidney, and large intestine were fixed in 3% glutaraldehyde in Sörensen's buffer at pH 6.8 for 24 h, dehydrated in ethanol and embedded in Epon 812 as usual (Glauert, 1975). Semithin (1–2 μm) sections were obtained with an Ultracut Reichert-Jung microtome, transferred to a drop of water on slides and then dried on a hot plate at 40 °C. Extraction methods for DNA were performed on blood smears by treatment with DNase I (Serva, 0.5 mg/ml in 1 mM $MgCl_2$ at 37 °C for 2 h), or 5% trichloroacetic acid (TCA) at boiling temperature for 15 min. Unstained smears and sections were also checked for autofluorescence.

Stock solutions (0.5 mg/ml) of the compounds (Fig. 1) obtained by synthesis as the free base (Knoll Laboratories, Madrid) were first made in acetic acid and then diluted with distilled water to the appropriate concentration. In all cases, final acetic acid concentration was 0.05%. Blood smears and Epon sections were stained, respectively, with 20 and 50 μg/ml solutions of the compounds for 5 min, washed in distilled water, air-dried, and directly observed under oil immersion in a Zeiss photomicroscope III equipped with the epifluorescence condenser III RS, and the filter sets for ultraviolet (UV, 365 nm), violet (405 nm), and violet-blue (436 nm) exciting light. Microfluorometric measurements were carried out on erythrocyte nuclei under 436 nm excitation, using the photometer head 03 (Zeiss), the HTVR 446 photomultiplier, and an uranyl glass as the emission standard. The highest fluorescence value was chosen as 100%, and all the results were expressed as the mean ± standard deviation (SD) of this value.

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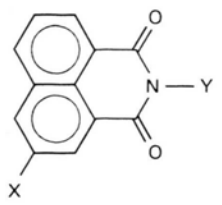

COMP.	X	Y	F.I. (±SD)
FA-142	$-NH_2$	$-CH_2CH_2N(CH_3)_2$	100 (4)
FA-2043	$-NH_2$	$-CH_2CH_2N(CH_2CH_3)_2$	100 (5)
FA-2143	$-NH_2$	$-CH_2CH_2N$ (pyrrolidine)	100 (3)
FA-655	$-OCH_3$	$-CH_2CH_2N$ (pyrrolidine)	40 (3)
M-12210	$-NO_2$	$-CH_2CH_2N$ (pyrrolidine)	20 (4)
M-4212	$-NO_2$	$-CH_2CH_2N(CH_3)_2$	30 (3)

Fig. 1. Chemical structure of the naphthalimide compounds (COMP.) used in this work. F. I.: quantitative measurements of the microscopic fluorescence intensity induced in chicken erythrocyte nuclei by treatment with the corresponding naphthalimides. Numbers correspond to the normalized values (mean ± standard deviation [SD]; $n = 10$).

Spectroscopic studies were performed on freshly made solutions (2 and 5 μg/ml in 0.05% acetic acid) of the highest fluorescent compounds (FA-142 [Amonafide], FA-2043 and FA-2143, see Fig. 1) using a Shimadzu UV-1601 spectrophotometer and a Perkin-Elmer 650–10S fluorescence spectrophotometer equipped with the R 372 F photomultiplier detector and 1 cm quartz cuvettes. The Raman scattering of solvents was subtracted from emission spectra.

After treatment of blood smears with the naphthalimide compounds, different fluorescence intensity was observed in erythrocyte nuclei (Fig. 1). The highest green-yellow emission was found using the 3-aminonaphthalimides FA-142, FA-2043, and FA-2143, whereas 3-methoxy (FA-655) or 3-nitro derivatives (M-12210 and M-4212 [Mitonafide]) showed very weak fluorescence. The strong emission of 3-aminonaphthalimides is explained because electron donors (such as the amino group) increase the fluorescence, while nitro and alkoxy substituents are electron accepting groups which decrease the emission intensity (Rhys Williams, 1980; Stockert *et al.*, 1994). The aliphatic side chain linked to the naphthalimide

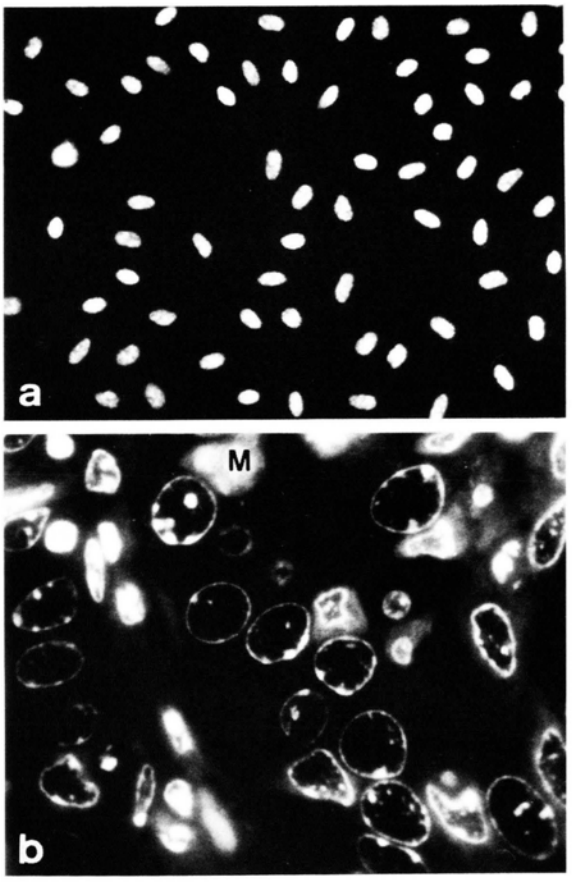

Fig. 2. Fluorescence patterns observed after treatment with FA-142. Exciting light: 436 nm. a: Chicken blood cells showing the emission of erythrocyte nuclei. X 500. b: Semithin Epon section of mouse large intestine showing the high fluorescence of compact chromatin masses. Mucin from goblet cells (M) also appears fluorescent. X 1000.

fluorophore did not appear relevant. Optimal excitations were 405 and 436 nm, and only a low fading rate occurred after prolonged irradiation.

Chromatin fluorescence induced by 3-aminonaphthalimides was abolished after DNA extraction by DNase or TCA. No emission was found in other cells or cell structures from naphthalimide-treated smears. In addition to erythrocyte nuclei (Fig. 2a), chromatin from tissue sections also showed strong fluorescence (Fig. 2b). The mucin content of goblet cells appeared with considerable emission, probably due to the remaining hydrophilia of acid glycosaminoglycans in plastic sections (Juarranz *et al.*, 1987; Tato *et al.*, 1991).

Spectroscopic studies revealed absorption peaks at 346 and 408 nm for FA-142, whereas the fluorescence emission appeared centered at 570 nm

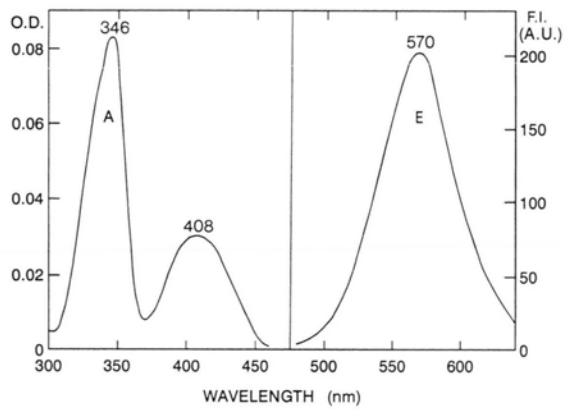

Fig. 3. Absorption (A), and fluorescence emission (E) spectra of the compound FA-142 (A: 5 μg/ml; E: 2 μg/ml). Exciting light: 440 nm. O. D.: optical density. F. I.: fluorescence intensity in arbitrary units (A. U.).

(Fig. 3). Other suitable excitations were 340, 380, 400 and 440 nm. Very similar absorption and emission spectra were also found for FA-2043 and FA-2143. Emission features of these naphthalimides appeared well correlated with the bright green-yellow fluorescence of chromatin under violet-blue (436 nm) excitation.

Taking into account the molecular structure of these compounds, chromatin fluorescence could be due to the intercalation of the naphthalimide fluorophore into DNA. Intercalative binding occurs for unsubstituted (Yen *et al.*, 1982), 3-amino (Hsiang *et al.*, 1989), and 3-nitro derivatives (Waring *et al.*, 1979; Chen *et al.*, 1993). The antitumoral FA-142 (Amonafide, currently used in clinical trials [Saez *et al.*, 1989]) was also shown to inhibit DNA topoisomerase II and to form an intercalator-stabilized topo II-DNA cleavable complex (Hsiang *et al.*, 1989).

In view of the wide use of fluorescence techniques in DNA cytochemistry and cell biology (Stockert *et al.*, 1990; Rye *et al.*, 1992; Stockert, 1992), investigations on new fluorescent probes are of considerable interest, particularly those with biological activity (Villanueva *et al.*, 1987; Stockert *et al.*, 1989, 1991; Molero *et al.*, 1995). The fluorescence emission of some antitumoral naphthalimides is a very useful property of these DNA-intercalating drugs, which could find important applications in biomedical research.

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