DNA affinity of new aminothioloxazolopyridocarbazole derivatives determined both in vitro and in single living cells

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Preliminary communication

DNA affinity of new aminothioloxazolopyridocarbazole derivatives determined both *in vitro* and in single living cells

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Summary — New potential DNA radioprotective agents were obtained by coupling an oxazolopyridocarbazole nucleus (NMHE) to simple aminothiol molecules such as cystine, cysteamine and WR2721. The ability of the new adducts to compete with ethidium bromide DNA binding was determined through their IC_{50} values which ranged between 1.4 and 2.75 x 10^{-6} mol·dm^{-3}, whereas for aminothiols IC_{50} ranged between 3 and 6 x 10^{-3} mol·dm^{-3}. Similarly, the apparent DNA-binding constants for aminothiol-OPCs were found to be 200–1000 fold higher than for parent molecules. The apparent DNA binding constants of the adducts was strongly influenced by the medium ionic strength, which suggests that ionic interactions occur in the overall binding process. Microspectrofluorometric analysis of drug intracellular localization in SC_{10} living cells revealed that aminothiol-OPCs were specifically accumulated in the cell nucleus.

DNA aminothiol radioprotectors / oxazolopyridocarbazoles / DNA affinity / DNA accessibility / microspectrofluorometry

Introduction

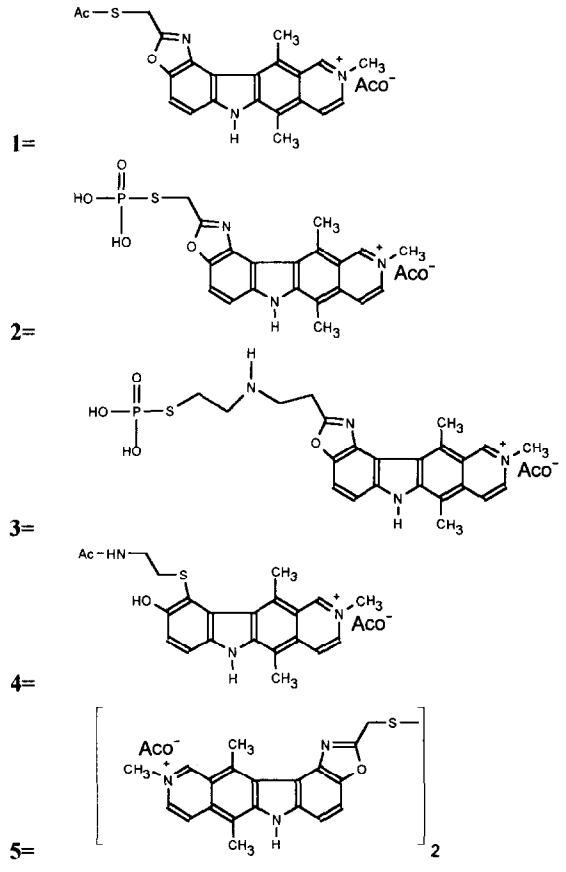
Some simple organic compounds whose structure is based on the aminothiol moieties (scheme 1) display *in vivo* radioprotective activity. These properties were first described for cystein and further generalized for other molecules endowed with the aminothiol group [1]. Meanwhile, all such drugs display an intrinsic toxicity and a short-lived radioprotective activity [2]. Recently, more efficient thiophosphorylized derivatives, such as WR2721, were proposed as prodrugs unmasking the aminothiol group in biological media [3]. As regards their possible molecular mechanism of activity, it has been suggested that these drugs could trap free radicals before they reach their biological target(s) [4]. Nevertheless, some recent work has demonstrated that a noticeable radioprotective effect could be achieved at drug concentrations lower than that necessary for total free-radical trapping [5]. On the other hand, Nakamura *et al* [6] have shown that the radioprotector effect is directly related to its DNA affinity. These results indicate the importance of the local drug concentration at the level of some fundamental biomolecules inside the cells; nuclear DNA being one of the most important. Hence, some previous attempts to improve radioprotector affinity for DNA have consisted of 'adding' a DNA intercalator with a high base-pair affinity, such as acridine, quinoline [7] or phenanthridine [8], to the radioprotective aminothiol group. Some of these molecules have been shown to display free-radical trapping at the level of DNA [9] but, to our knowledge, the DNA-binding properties of these molecules have not yet been investigated.

In the present study, our aim was to determine the potential increase in DNA binding obtained by coupling an oxazolopyridocarbazole (OPC) nucleus to simple aminothiol molecules such as cystine, cystamine and WR2721 (scheme 1). The OPC nucleus was chosen because of its high affinity for DNA [10] and its relatively low cytotoxicity [11, 12] and mutagenicity [13, 14]. The DNA-binding constants of aminothiol-OPCs and the parent compounds (scheme 1) were determined from their ability to compete with ethidium bromide. These experiments showed that the higher binding constants observed with aminothiols were only 3700 mol·dm^{-3} whereas, as expected, aminothiol-OPCs displayed higher affinity constants in the 1 to 5 x 10^5 mol·dm^{-3} range, values similar to those previously observed for the unsubstituted OPC nucleus [10]. Moreover, competition experiments between aminothiol-OPCs and aminothiols were performed and showed that, even at high concentrations, simple aminothiols failed to displace aminothiol-OPCs from their DNA binding sites.

L-HOOC-CH(NH2)-CH_2-S-S-CH2-CH(NH2)-COOH **Cystine**

H2N-CH2-CH2-S-S-CH_2-CH_2-NH_2 **Cystamine**

H_2N-CH_2-CH_2-CH_2-NH-CH_2-CH_2-S-PO_3H_2 **WR2721**



Scheme 1. Structures of aminothiol and synthesized aminothiol-OPC molecules.

Aminothiol-OPCs showed interesting fluorescence properties with an emission band located at 520 nm and a high fluorescence quantum yield. These spectroscopic properties allowed the study of their subcellular localization using the newly developed microspectrofluorometry technique [15]. Microspectrofluorometric experiments performed with SC_{10} cells showed the specific localization of the aminothiol-OPC adducts within the nucleus.

Comparison between DNA-binding parameters for aminothiols and aminothiol-OPCs observed both *in vitro* and in single living cells showed that addition of an OPC nucleus to the aminothiol moiety leads to a marked increase in DNA affinity.

Results and discussion

As a first step, the relative DNA affinity of aminothiols and aminothiol-OPCs were compared by their ability to compete with ethidium bromide binding [16, 17]. The molecules under investigation can be ranked by their IC_{50} values, *ie* the micromolar concentration displaces 50% of bound EB. For classical aminothiol

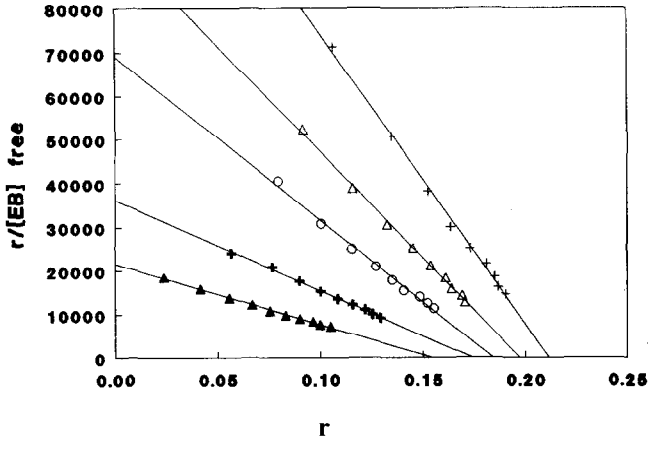


Fig 1. Scatchard isotherms of ethidium bromide binding to calf thymus DNA in the presence of increasing concentration of **5** at 0.15 mol·dm^{-3} NaCl: + 0; Δ 0.8; O 1.6; ✚ 6.1 and ▲ 12.3 x 10^{-6} mol·dm^{-3}.

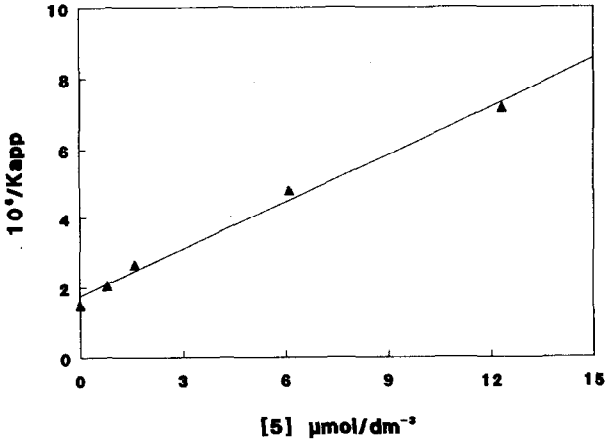


Fig 2. Variation of $1/K_{aap}$ *versus* concentrations of **5** in 0.15 mol·dm^{-3} NaCl medium.

Table I. Affinity constants and IC_{50} values of aminothiol-OPCs and simple aminothiols.

Compound	*Affinity constant K x 10^5*					*IC_{50} x 10^{-6}*
	Ionic strength [NaCl] (mol·dm^{-3})					*(mol·dm^{-3})*[a]
	0.25	*0.15*	*0.10*	*0.075*	*0.038*	
1	0.45	0.91	1.12	1.14	2.02	2.75
2	0.43	0.77	1.29	1.43	3.09	1.86
3	1.11	1.70	3.92	2.57	7.53	1.42
4	1.18	1.50	1.35	3.23	4.07	1.99
5	0.98	2.10	5.39	11.7	34.7	1.72
EB	3.23	5.75	7.41	9.62	20.0	–
Cystine			0.0037			6000
Cystamine			0.0001			3140
WR2721			0.0018			3686

[a]Measured at ionic strength 0.10 [NaCl] mol·dm^{-3}.

radioprotectors, the IC_{50} values range between 3 and 6 x 10^{-3} mol·dm^{-3}, which indicates a relatively low aptitude for competition with ethidium binding. In the case of aminothiol-OPCs, IC_{50} values range between 1.4 and 2.75 x 10^6 mol·dm^{-3}, which suggests that the adducts were 500- to 1000-fold more efficient in EB displacement than their parent counterparts. In order to evaluate more precisely the drugs' association constants with DNA, ethidium bromide Scatchard isotherms [18] were measured in the presence of different concentrations of radioprotectors. As an example, the Scatchard plots of EB alone and in the presence of 5 different concentrations of compound **5** are given in figure 1. The variation of the apparent binding constant of EB in the presence of aminothiol-OPC competitor allows us to determine the DNA-binding constant of the competitor from the slope of the straight line obtained when plotting $1/K_{app}$ *versus* drug concentrations [17] (fig 2). The binding constants inferred from this method are given in table I for comparison. As previously observed for the IC_{50} values, the affinity constants for coupled molecules were found to be largely higher than those obtained with parent molecules (from 200- to 1000-fold, *cf* table I).

In the case of compound **1**, the intrinsic spectroscopic properties of the drug allowed us to perform direct titration experiments from its fluorescence enhancement upon DNA binding. The Scatchard plot obtained with this molecule shows a linear behaviour suggesting the presence of a unique class of binding site with an affinity constant of 3.6 x 10^5 mol^{-1}·dm^3 and a maximum number of bound molecules per phosphate of 0.12, which corresponds to 1 bound molecule for *ca* 8 bases. The fluorescence increase of compound **1** upon DNA binding was also used to perform competition experiments with uncoupled aminothiols, in which it was used as a fluorescent probe. As shown in figure 3, concentrations of WR2721 as high as 0.6 x 10^{-3} mol·dm^{-3} only led to a weak modification of the apparent binding constant of compound **1**. This confirms that aminothiol-OPCs have a DNA affinity that is 2 orders of magnitude higher than simple aminothiols.

In order to evaluate the influence of the medium ionic strength on the binding constants [19, 20], binding experiments were also carried out in the

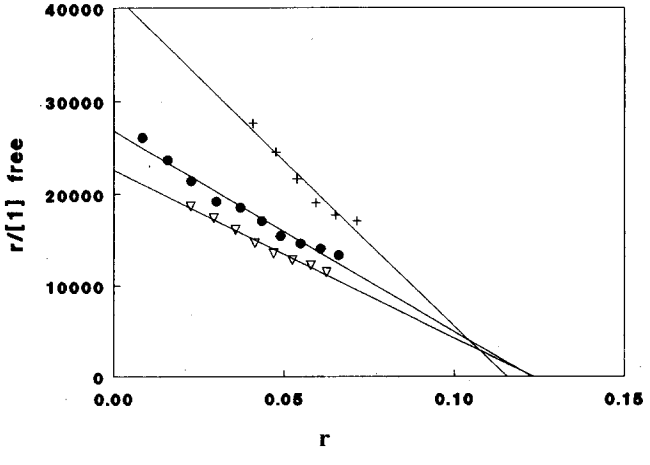


Fig 3. Scatchard isotherms of compound **1** binding to calf thymus DNA in the presence of increasing concentrations of WR2721 at 0.1 mol·dm^{-3} NaCl: + 0; ● 0.304 and Δ 0.608 x 10^{-3} mol·dm^{-3}.

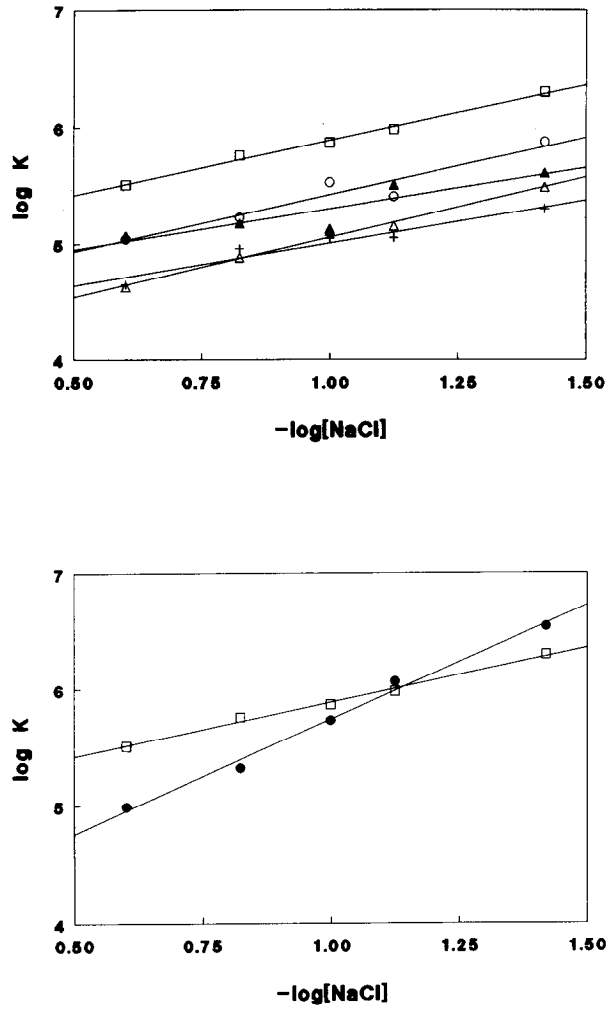


Fig 4. a) Variation of log *K versus* log[NaCl] for monocationic aminothiol-OPCs in comparison with ethidium bromide. +, compound **1**; Δ compound **2**; O compound **3**; ▲ compound **4**; □ ethidium bromide. b) Variation of log *K versus* log[NaCl] for the dicationic aminothiol-OPC; compound **5** ●; compared with ethidium bromide □.

presence of increasing NaCl concentrations (from 0.038 to 0.25 mol·dm^{-3}). As shown in figure 4a and b, where log *K* is plotted *versus* log [NaCl], a slope of about −1 was observed with monocationic aminothiol-OPC derivatives, whereas for the dicationic **5** a slope of about −2 was observed. These results suggest that ionic interactions between the drugs cationic charge and the DNA phosphates strongly influence the binding process [19, 20]. Similar variations with ionic strength were previously observed for other OPC-substituted compounds [10].

In order to study the intracellular distribution of aminothiol-OPCs, SC_{10} cells were incubated in the presence of 0.5 x 10^{-6} mol·dm^{-3} aminothiol-OPCs for 1 h. The fluorescence emission spectra of aminothiol-OPCs were recorded in different compartments of SC_{10} cells. For all studied aminothiol-OPCs, fluorescence was only detected in the nucleus, as shown in figure 5 in the case of the compound **1**; a very weak fluorescence emission was detected when the laser beam was focused in the cytoplasm (spectrum **a**). On the contrary, a high fluorescence emission was detected from a selected microvolume within the cell nucleus (spectrum **b**). Moreover, spectrum **b** was identical, when corrected from the cell intrinsic fluorescence, to the emission spectrum recorded when the drug was bound to DNA *in vitro* (spectrum **c**). Some other experiments were performed in order to determine if the very low fluorescence detected from cytoplasm (fig 5, spectrum **a**) could result from a quenching of the drug fluorescence in the presence of some cytoplasmic components. Nuclei were isolated from SC_{10} cells [21] and the fluorescence emission spectra of the drugs were performed in the presence of either isolated nuclei or the supernatant obtained after nucleus isolation. These fluorescence measurements revealed an increase of the drug fluorescence quantum yield in the presence of nuclei and of cytosolic components (fig 6), thus suggesting that the very low fluorescence detected from cytoplasm was not related

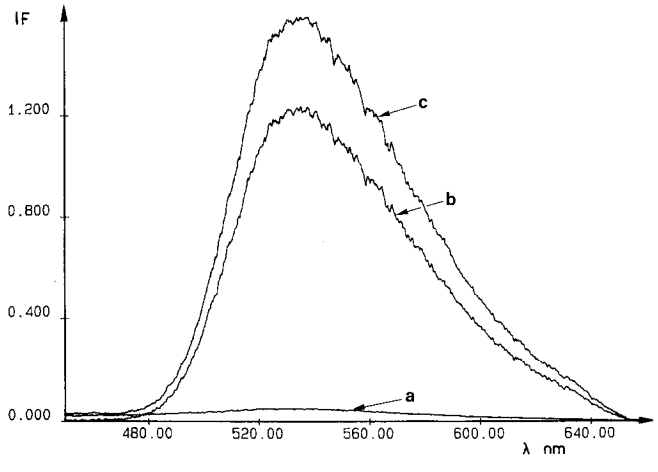


Fig 5. Microspectrofluorometric experiments. Fluorescence emission spectra (λ_{ex} = 351 nm) recorded in single living SC_{10} cells incubated in the presence of 0.5 x 10^{-6} mol·dm^{-3} of compound **1** for 1 h: spectrum **a** selected fluorescence emission from the cytoplasm; spectrum **b** selected fluorescence emission from the nucleus; and spectrum **c** fluorescence emission (λ_{ex} = 351 nm) of a 0.5 x 10^{-6} mol·dm^{-3} aqueous solution of compound **1** in the presence of 50 x 10^{-6} mol·dm^{-3} calf thymus DNA.

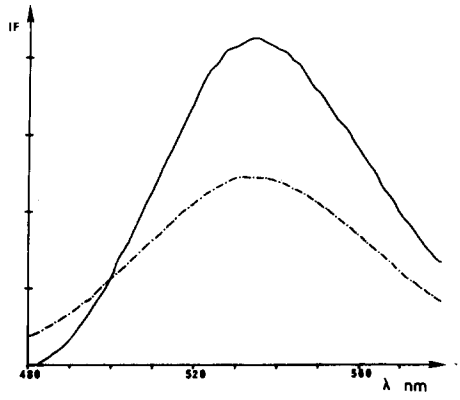


Fig 6. Fluorescence emission spectra (λ_{ex} = 305 nm) 10^{-6} mol·dm^{-3} compound **1** in the supernatant obtained after nucleus isolation diluted (1/50) in cacodylic buffer pH 7.4 (——) and in cacodylic buffer pH 7.4 (–·–·–).

to a quenching effect but actually reflects a very low drug concentration in the cytosol. The microspectrofluorometric data obtained within SC_{10} cells can therefore be attributed to the high drug affinity for nuclear DNA.

Taken together, the results obtained both *in vitro* and in single living cells suggest that addition of an OPC chromophore to the aminothiol moiety leads to new derivatives endowed with high DNA-binding potencies which improve their accessibility for nuclear DNA within living cells. The present study can be considered as a preliminary step towards the rational design of DNA radioprotectors. Nevertheless, in order to achieve some true DNA radioprotective activity, it is necessary to determine whether aminothiol-OPCs still display radical-trapping properties when OPC bound.

Experimental protocols

Synthesis and purification

Aminothiol-OPCs were synthesized according to a previously published procedure [22, 23], as shown in scheme 2. Briefly, they were obtained from enzymatic oxidation (H_2O_2–horseradish peroxidase system) of 9-hydroxyellipticinium followed by either amino or thiol nucleophilic attack at C(10). In the case of amine a cyclization process occurs between O(9) and N(10) atoms [24, 25].

All molecules were purified by liquid chromatography on a Biorad SM_2 hydrophobic column as solid phase using a 2–50% ammonium acetate (pH 5)/methanol gradient as a liquid phase. Samples of drugs were routinely checked for purity by both thin-layer chromatography on silica-gel plates, in a solvent system containing butanol/acetic acid/H_2O (5:1:1) and HPLC using a Waters apparatus equipped with a reversal microbondapack column and ammonium acetate/methanol (70:30) as the eluent.

DNA binding experiments

Fluorescence titration experiments were performed with a computer-controlled Perkin-Elmer LS 50 spectrometer in quartz fluorescence cells (1 cm pathlength) containing 3 cm^3 of buffered solution. In the case of compound **1**, the intrinsic fluorescence increase of the drug upon DNA binding was used for direct titration experiments. With other aminothiol-OPCs, the low fluorescence increase upon DNA binding rendered difficult direct titrations and binding parameters were obtained *via* ethidium bromide (EB) competition experiments [16, 17].

Direct binding fluorescence experiments

Addition of DNA to a solution of compound **1** leads to a marked increase in fluorescence intensity associated with a red shift of the excitation spectrum. The optimal detection wavelength, corresponding to the highest differences in fluorescence intensity between free and bound drugs, was λ_{ex} = 340 nm and λ_{em} = 530 nm. The concentration of bound drug, *Cb*, was calculated according to the following equation [10]:

$$Cb = \Delta I_f/[k(V - 1)]$$

Where ΔI_f is the difference between the fluorescence of free and bound drug, V is the ratio of the limit fluorescence of free and bound drug, and k is a factor that correlates free drug concentration to fluorescence intensity.

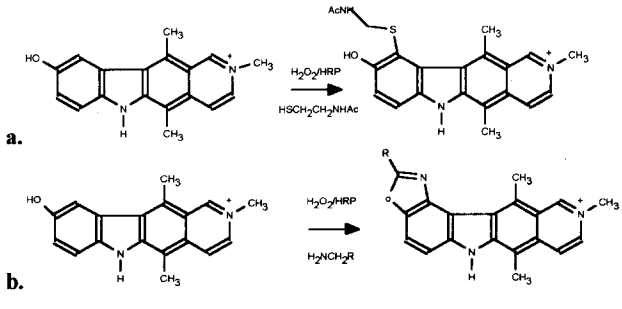
Ethidium bromide competition experiments

The DNA binding parameters of drugs were determined using their ability to compete with the binding of ethidium bromide. IC_{50} is the micromolar drug concentration that displaces 50% of 10^{-6} mol·dm^{-3} of ethidium bromide initially bound to 20 x 10^{-6} mol·dm^{-3} calf thymus DNA. This was determined according to Bagulay *et al* [16].

Apparent binding constants of aminothiol-OPCs adducts to DNA were determined from the variation in the apparent binding constant of ethidium bromide with DNA in the presence of increasing concentrations of the tested compounds [17].

Cells

SC_{10} is an human bronchic cell line, which we obtained from MF Poupon (Institut Curie, Paris, France). Cells were grown in a humidified 5% CO_2 atmosphere, in RPMI 1640 (Bohringer) supplemented with 10% heat-inactivated foetal calf serum, 2 x 10^{-3} mol·dm^{-3} L-glutamine, streptomycin 0.1 mg/cm^3 and penicillin (100 U/cm^{-3}). Before microspectrofluorometric analysis, cells were treated by trypsin/EDTA and seeded in petri dishes.



Scheme 2. Synthesis procedure of: a) compound **3**; and b) other OPC compounds.

Microspectrofluorometric analysis

The apparatus used has been described previously [15]. Briefly, it is composed of a Zeiss UMSP 80 UV epifluorescence microscope, optically coupled by UV reflecting mirrors to a Jobin-Yvon spectrograph. The 351 nm UV line of an argon laser (Spectra Physics) was used for the excitation of the drug. The laser beam was focused on the cell through the microscope objective on a circular spot of 1 μm diameter. The excitation power was reduced to 1 μW by optical neutral density filters. Fluorescence spectra were recorded in the 400–650 nm region on a 1024 diode-intensified optical multichannel analyzer (Princeton Instruments) with a resolution of 0.25 nm/diode. The data were stored and processed on a 80286 IBM PS/2 microcomputer using Jobin-Yvon 'Enhanced Prism' software. Cellular fluorescence spectra were obtained with a X100 Zeiss ultrafluar objective (NA = 1.32). In order to avoid any possible fluorescence from a plastic or glass support during analysis with near UV excitation, cells were disposed on a quartz plate on the microscope stage in 100 mm thermostated petri dishes, filled with 5 ml phosphate buffer saline. The objective was immersed in culture medium and a circular area of 2 μm diameter was analysed in the nucleus or in the cytoplasmic region by interposing a field diaphragm within the microscope on the emission pathway. The accumulation time of each spectrum proceeded for 1 to 10 s. Sample heating, photobleaching and photodamage were checked empirically and found to be negligible under our experimental conditions. In particular, cells always remained viable after repeated fluorescence determinations, as checked by phase-contrast microscopy.

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