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The Bcl-2 Antagonist HA14-1 Forms a Fluorescent Albumin Complex that Can Be Mistaken for Several Oxidized ROS Probes

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Abstract

The proapoptotic effects of the Bcl-2 antagonist HA14-1 are believed to derive from its affinity for the hydrophobic groove on Bcl-2 and Bcl-x_L, thereby displacing proapoptotic factors, *e.g.* Bax and Bak. We have reported that HA14-1 promotes the efficacy of low-dose photodynamic therapy (PDT). A recent report proposed that the proapoptotic activity of HA14-1 reflects its ability to generate reactive oxygen species (ROS) when incubated in an aqueous environment. This later study, like several other HA14-1 investigations, relied on the use of fluorescent probes for ROS detection. We found that HA14-1 reacts with the albumin in serum to yield a fluorescent product. After correcting for this effect, the putative formation of ROS by HA14-1 could not be demonstrated with the fluorescent probes H₂DCFDA, dihydroethidium or dihydrorhodamine. Indeed, the fluorescence excitation/emission spectra of HA14-1 encompassed the excitation/emission wavelengths used to detect these ROS probes. Cells cultured in a medium supplemented with ovalbumin, instead of serum, underwent apoptosis following HA14-1 addition, but did not exhibit fluorescence. Hence, HA14-1 fluorescence was unrelated to its proapoptotic activity. We conclude that the enhancement of PDT by HA14-1 reflects a pharmacologic effect, rather than its direct contribution of ROS.

INTRODUCTION

The Bcl-2 antagonist ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (HA14-1) was initially identified by screening for agents that could fit into a hydrophobic binding pocket on Bcl-x_L (1), and thereby inactivate antiapoptotic functions of this and other members of the Bcl-2 family. Since that report, there have been over 60 publications, including several from our laboratory (2,3), indicating the development of apoptosis in mammalian cells following exposure to HA14-1. Synergism between HA14-1 and chemotherapeutic agents has also been reported (2,4–8). In addition, we have reported that exposure of murine leukemia L1210 cells to HA14-1 also activates autophagy (3). This is consistent with reports, mainly from Levine's group, that the proautophagic protein Beclin-1 is "inactivated" as a consequence of binding to Bcl-2 family proteins (9). Displacement of Beclin-1 by HA14-1 could provide a mechanism for initiation of autophagy.

We (10) and Oleinick's group (11) have shown that antiapoptotic Bcl-2 family proteins are targets for photodynamic therapy (PDT), and that their PDT-induced inactivation/loss often correlates with the initiation of apoptosis. In a recent report, we established that photokilling by low-dose PDT could be markedly enhanced by a concentration of HA14-1 that, by itself,

had only a minor proapoptotic effect (12). These findings suggested that HA14-1 was acting independently of PDT, possibly by inactivating Bcl-2 and related proteins, and thus reducing the threshold needed for the induction of apoptosis.

It is known that HA14-1 is unstable in an aqueous environment (13,14). In a recent study, Doshi *et al.* (14) monitored the disappearance of HA14-1 in culture medium and the appearance of a series of decomposition products. The calculated half-life of HA14-1 was 15 min. In this latter study, the disappearance of HA14-1 correlated with the oxidation of 2', 7'-dichlorodihydrofluorescein (H₂DCF) to DCF (dichlorofluorescein) in both culture medium and cell culture. Inclusion of the antioxidants *N*-acetylcysteine (NAC) or vitamin E suppressed both H₂DCF oxidation and the development of apoptosis in cell culture. As H₂DCF oxidation is widely employed for the monitoring of reactive oxygen species (ROS), Doshi *et al.* (14) proposed that the proapoptotic effects of HA14-1 were a consequence of the oxidative stress induced by agent-derived ROS. Other investigators, using similar approaches, have also concluded that ROS formation occurs following the treatment of cultured cells with HA14-1 (5–8,15).

In this study, we examined the potential role of ROS formation induced by HA14-1 as a factor in the initiation of apoptosis. We found that the fluorescence attributed to H₂DCF oxidation actually reflected a fluorogenic interaction between HA14-1 and the albumin component of serum, and was unrelated to the generation of ROS, or the presence of the ROS probe.

MATERIALS AND METHODS

Chemicals and biologicals

Amino acids, tissue culture medium, *N*-acetyl cysteine, ovalbumin, albumin and γ -globulin were purchased from Sigma-Aldrich (St. Louis, MO). Sterile horse serum was provided by Atlanta Biologicals (Lawrenceville, GA). HA14-1 was obtained from Ryan Scientific, Inc. (Isle of Palms, SC). Solutions were made up in anhydrous dimethyl sulfoxide and stored in small aliquots at -20°C . Fluorescent probes were purchased from Molecular Probes (Eugene, OR). These included dihydrorhodamine (DHR, a probe for H₂O₂), dihydroethidium (DHE, a probe for superoxide anion), DEVD-R110 and the diacetate of H₂DCF (H₂DCFDA). H₂DCF was prepared by alkaline hydrolysis of H₂DCFDA (14).

Cells and maintenance

Murine leukemia L1210 cells were grown in a modification of the α -MEM formulation (Sigma-Aldrich) previously described (3). Unless stated otherwise, all studies described herein were carried out in "MEMH," a modified α -MEM formulation supplemented with 20 mM HEPES pH 7.4 (replacing NaHCO₃), along with 10% horse serum.

DEVDase activity

Activation of procaspases-3 and -7 was assessed by measuring hydrolysis of the fluorogenic substrate DEVD-R110 (16) 30 min after addition of HA14-1 to cell cultures. This substrate releases the fluorescent dye Rhodamine 110 upon enzymatic hydrolysis. The fluorogenic response was measured with a Fluoreskan fluorescence plate reader using 485 nm excitation and 510 nm emission. The procedure is outlined in Ref. (2). In some studies, HA14-1 was first incubated with MEMH prior to addition to cell culture. The BioRad assay, using BSA as a standard, was used to estimate protein concentrations.

Fluorescence detection of ROS and HA14-1 / albumin complexes

An SLM 48000 fluorometer, with electronics modified by ISS (Champaign, IL), was used in the slow-kinetic mode to monitor HA14-1 and ROS probe-derived fluorescence. Data points

were acquired every 3 or 6 s for 3–6 min, unless otherwise specified. Slit widths of 2 nm (excitation) and 4 nm (emission) were employed. Excitation and emission wavelengths were: H₂DCFDA and H₂DCF, 490/520 nm; DHE, 518/605 nm; DHR, 490/530 nm; and HA14-1, 460/565 nm.

The fluorescence of HA14-1 and ROS probes was determined in the presence and absence of cells. The cell-free systems contained MEMH, or PBS (pH 7), or PBS + 10% horse serum. In the cell-free systems the ROS probes (10 μ M) were added just before the HA14-1. When cells were employed, suspensions of L1210 cells were exposed to 10 μ M of ROS probes for 30 min at 37°C in MEMH. Cells were subsequently collected by centrifugation, washed, and then resuspended in MEMH or MEMH in which the 10% horse serum was replaced with purified bovine albumin, or the other proteins listed in Table 1. HA14-1 was subsequently added.

RESULTS

Conditions used to monitor ROS probe fluorescence fall within the fluorescence excitation/emission spectra of HA14-1

We initially analyzed the fluorogenic response of cells loaded with H₂DCFDA upon addition of HA14-1. Data shown in Fig. 1A demonstrate a time-dependent increase in fluorescence upon addition of 25 μ M HA14-1 to cell cultures. However, a similar effect was observed with cells that contained no H₂DCFDA (Fig. 1A). We then examined the fluorescence of HA14-1 in a cell-free system containing H₂DCF + MEMH, HA14-1 + MEMH, or H₂DCF + MEMH + HA14-1 (Fig. 1B). Fluorescence emission from HA14-1 was observed whether or not H₂DCF was present, but not from H₂DCF alone. Both the kinetics of appearance and the relative amounts of fluorescence were nearly identical in MEMH containing HA14-1 or HA14-1 + H₂DCF.

Subsequent studies utilized cells that were loaded with DHE or DHR, probes for superoxide and H₂O₂, respectively (17). Analyses using excitation/emission wavelengths appropriate for oxidized DHE (518/605 nm) or DHR (490/530 nm) also revealed the development of fluorescence in HA14-1 treated cultures. However, comparable fluorescence occurred in HA14-1-treated cultures that lacked DHE or DHR (Fig. 1C).

An examination of the fluorescence parameters of HA14-1 in MEMH, in the absence of any fluorescent ROS probes, revealed a broad excitation peak centered at 460 nm, and an equally broad emission peak centered at 565 nm (Fig. 1D). These peaks are sufficiently broad so as to encompass the excitation and emission wavelengths used in the monitoring of H₂DCF, DHE and DHR oxidation. Hence, the HA14-1-dependent fluorescence observed in our system is not attributable to ROS-mediated probe oxidation. Instead, the observed fluorescence is derived from excited HA14-1 molecules.

Modulation of HA14-1 fluorescence by NAC and serum

The ability of NAC to suppress HA14-1-induced H₂DCFDA oxidation has been interpreted as evidence supporting HA14-1 production of ROS (14). The data presented in Fig. 2A indicate that this observation probably reflects the effects of NAC on HA14-1 fluorescence, and not H₂DCFDA. Specifically, addition of 5 mM NAC to MEMH prior to the addition of HA14-1 (in the absence of any ROS probe) suppressed the appearance of fluorescence in a cell-free system. Furthermore, the fluorescence generated by preincubation of HA14-1 in MEMH was quickly reduced by the addition of NAC (Fig. 2A).

Surprisingly, the addition of 25 μ M HA14-1 to PBS did not result in a fluorogenic effect (Fig. 2B). However, presence of 10% horse serum resulted in a rapid increase in fluorescence at 565 nm (Fig. 2B, Table 1). In other studies, we found that this serum effect could be mimicked by

the addition of human or bovine albumin, but not by ovalbumin or γ -globulin (Table 1), or by cytochrome *c*, ribonuclease, or myoglobin (D. Kessel, unpublished).

Significance of HA14-1 fluorescence to its proapoptotic activity

Maximum fluorescence was observed within 2 min of addition of HA14-1 to MEMH (Fig. 1B and Fig. 2B,C). Thereafter, there was a gradual loss of fluorescence with time (Fig. 2C). Storage of HA14-1 in MEMH for > 3 min before its addition to cultured cells suppressed the ability of the drug to induce DEVDase activity, a measure of procaspase 3/7 activation (Fig. 2D). The similarities in the kinetics of loss of fluorescence vs loss of proapoptotic activity initially suggested a relationship between these phenomena (compare Fig. 2C with Fig. 2D). To determine whether the observed fluorescence was critical to the proapoptotic activity of HA14-1, we performed DEVDase analyses on HA14-1-treated cells incubated in MEMH containing ovalbumin or γ -globulin, instead of horse serum. Neither ovalbumin nor γ -globulin promoted the development of HA14-1 fluorescence. However, both supported the activation of DEVDase (Table 1). This result indicates that the fluorogenic interaction that occurs when HA14-1 is added to the growth medium is unrelated to the proapoptotic activity of the agent.

The fluorescent product generated in the DEVDase assay is excited, and emits at wavelengths (excitation = 485 nm, emission = 510 nm) that are contained in the HA14-1 fluorescence spectrum (Fig. 1D). Hence, the possibility arose that HA14-1 fluorescence might inadvertently contribute to the signal detected in the DEVDase assay. However, this was not the case. Extracts of HA14-1-treated cells, in the absence of exogenous DEVDase substrate, yielded a fluorescent signal that was less than that measured in control extracts incubated with the DEVDase substrate (Table 1).

DISCUSSION

Several small fluorescent molecules (*i.e.* DHR, DHE and H₂DCFDA that undergo intracellular metabolism to yield H₂DCF) are routinely used as probes to monitor ROS generation and oxidative stress in cultured cells. The use of such probes has led several investigators to conclude that cells generate ROS following exposure to HA14-1 (5–8,14). Indeed, a recent study suggested that HA14-1 itself, upon interaction with an aqueous environment, generated ROS (14). We also observed that fluorescence is generated following exposure of ROS probe-loaded L1210 cells to HA14-1, or following exposure of H₂DCF to HA14-1 in a cell-free system. However, we also showed that HA14-1 was fluorogenic, and that the observed fluorescence in our systems was HA14-1 dependent, and ROS probe independent. Fluorescence occurred because the conditions used for ROS probe excitation and monitoring fell within the fluorescence spectra needed for HA14-1 excitation and emission. Although our studies indicate that proapoptotic concentrations of HA14-1 neither directly generate ROS, nor promote ROS generation in L1210 cells, we cannot discount the claims made by other laboratories, in other cell types, that HA14-1 promotes ROS generation or accumulation (5–8). Nevertheless, our data emphasize a potential confounder in the use of fluorescent ROS probes as monitors of HA14-1-induced oxidative stress, and indicate a critical control needed for meaningful interpretation of experimental data.

HA14-1 fluorescence required its incubation with either serum or albumin. Neither γ -globulin nor ovalbumin could substitute for albumin. The capacity of albumin to bind proteins and small molecules is well documented, as is its potentiation of the fluorescence of some bound molecules (18–21). HA14-1 has several structural features compatible with its binding to drug-binding site-1 on human albumin (20). In addition, Xing's group has characterized two analogs of HA14-1 termed 3e and sHA14-1, which are identical except for the presence of a nitrile group in 3e (14,22). Whereas 3e generates a fluorescent signal when incubated with H₂DCF, sHA14-1 does not (14,22). If one assumes that the fluorescence attributed to the HA14-1 analog

3e actually reflects 3e binding to albumin, it appears that the nitrile group in HA14-1 may be critical for its binding to albumin. Indeed, addition of a nitrile group to gossypol markedly enhances its binding to albumin (23).

The fluorescence observed following HA14-1 and serum interaction decayed rapidly, and paralleled the agent's ability to induce apoptosis (compare Fig. 2C with Fig. 2D). Doshi *et al.* (14) reported a half-life of ~15 min for HA14-1 in aqueous environments, and showed that the decomposition products of HA14-1 are neither proapoptotic nor support the development of fluorescence when mixed with H₂DCF. This latter group also reported that NAC did not alter the half-life of HA14-1 in aqueous medium, but did decrease the fluorogenic effect. In view of the data shown in Fig. 2A, we suggest that NAC either quenches the fluorescence of the HA14-1/albumin complex, or causes its disruption. The studies with ovalbumin or γ -globulin reported in Table 1 clearly indicate that HA14-1 related fluorescence is not critical to the proapoptotic activity of the molecule. Whether the association of HA14-1 with albumin offers some tangible biologic effect (enhanced stability, facilitates cellular uptake, *etc.*) is not known.

HA14-1 is increasingly being used in combination with a second therapeutic to treat cancers. Several examples exist in which HA14-1 plus second agent co-treatment enhanced ROS probe fluorescence markedly above that observed with either agent alone (4–8). In such protocols, HA14-1 might have enhanced ROS production. We recently reported that low-dose HA14-1 co-treatment markedly enhances PDT photokilling (12). Although our current studies indicate that HA14-1 itself does not generate ROS, it is unclear as to whether the synergistic combinational effects reflect a pharmacologic inactivation of antiapoptotic Bcl-2 protein by HA14-1, and hence a lowering of the threshold needed for the induction of apoptosis, and/or an amplification of the toxic ROS species generated during PDT. This issue is currently under investigation.

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Figure 1.

(A) Fluorescence emission by L1210 cells as a function of time. ($\blacksquare$) Cells preloaded with H₂DCFDA, then exposed to 25 μ M HA14-1; ($\square$) cells exposed to 25 μ M HA14-1 with no H₂DCFDA present. Excitation = 490 nm, emission = 520 nm. (B) H₂DCF fluorescence (EX = 490 nm, EM = 520 nm) in a cell-free system containing MEMH (+) or MEMH + 25 μ M HA14-1 ($\square$). The fluorescence of HA14-1 in MEMH in the absence of H₂DCF is also shown ($\bullet$). (C) Fluorescence of 25 μ M HA14-1 in L1210 cells in the absence ($\circ$) vs presence ($\bullet$) of DHE (EX = 518 nm, EM = 605 nm); in the absence (Δ) vs presence ($\blacktriangle$) of DHR (EX = 490 nm, EM = 530 nm). (D) Fluorescence emission and excitation spectra of 25 μ M HA14-1 in MEMH.

Figure 2.

(A) Fluorescence of 25 μM HA14-1 in MEMH (cell free) at 565 nm using 460 nm excitation (▲), effects of adding 5 mM NAC after 120 s (Δ) in MEMH with 5 mM NAC added at the beginning of incubation (+). (B) Fluorescence of 25 μM HA14-1 in PBS with 10% serum added after 180 s; (C) decrease in fluorescence of 25 μM HA14-1 in MEMH over a 15 min interval (EX = 460, EM = 565); (D) DEVDase activity in L1210 cells 30 min after addition of 25 μM (●) or 50 μM (○) of freshly prepared HA14-1, or drug stored in MEMH for the specified times shown on the x-axis.

Table 1

DEVDase activation and fluorogenic effects elicited by different proteins.

Protein	Conditions	DEVDase *	Fluorescence emission [†]
10% horse serum	HA14-1 omitted	0.10 ± 0.03	0.15 ± 0.07
10% horse serum		4.4 ± 0.3	10.5 ± 0.7
Human albumin		4.0 ± 0.4	8.1 ± 0.5
Bovine albumin		3.9 ± 0.2	9.4 ± 0.2
Ovalbumin		4.7 ± 0.4	0.52 ± 0.2
γ-Globulin		3.5 ± 0.3	0.21 ± 0.04
10% horse serum	DEVD-R110 omitted	<0.05	—

* nmol product min⁻¹ mg⁻¹ protein.

[†] Fluorescence at 565 nm upon excitation at 460 nm. Cells were incubated at 37°C in MEMH containing 10% horse serum, or with serum replaced by 4 mg mL⁻¹ of each protein specified. HA14-1 (25 μM) was added to all tubes but the control. Samples were removed 3 min later for analyses of fluorescence emission at 565 nm (excitation = 460 nm). Additional samples were removed 30 min after HA14-1 addition for analyses of DEVDase activity. The substrate (DEVD-R110) was omitted from one tube as specified. Data represent mean ± SD for three determinations.