

## Type 1 Phototherapeutic Agents. 2. Cancer Cell Viability and ESR Studies of Tricyclic Diarylamines

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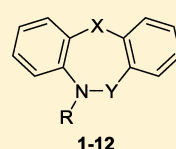
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### S Supporting Information

**ABSTRACT:** Type 1 phototherapeutic agents based on diarylamines were assessed for free radical generation and evaluated in vitro for cell death efficacy in the U937 leukemia cancer cell line. All of the compounds were found to produce copious free radicals upon photoexcitation with UV-A and/or UV-B light, as determined by electron spin resonance (ESR) spectroscopy. Among the diarylamines, the most potent compounds were acridan (4) and 9-phenylacridan (5), with IC<sub>50</sub> values of 0.68  $\mu$ M and 0.17  $\mu$ M, respectively.

**KEYWORDS:** Diarylamines, ROS, type 1, type 2, photosensitizers, radicals, ESR



X = Single Bond,  
CH<sub>2</sub>, O, S, CO,  
CH<sub>2</sub>CH<sub>2</sub>,  
CH=CH, or  
N=N.

Y = Single Bond or CH<sub>2</sub>.  
R = H or CH<sub>3</sub>.

The hallmark of phototherapy is its ability to destroy lesions with minimal or negligible effect on normal tissues. Furthermore, the cosmetic effect achieved after phototherapy of head and neck and skin cancers has been remarkable.<sup>1,2</sup> As is commonly known, phototherapeutic agents operate via two principal mechanisms, type 1 and type 2,<sup>3–5</sup> and all the clinically used photosensitizers operate via a type 2 process mediated by singlet oxygen. We have been directing our efforts on the development of novel type 1 phototherapeutic agents to complement the currently used type 2 (PDT) agents and enhance the phototherapy arsenal. Type 1 agents encompass wider classes of compounds than those of type 2 because type 1 photosensitizers can cause cell death either via direct interaction of the excited photosensitizer with the tissue or indirectly through the generation of secondary reactive oxygen species (ROS) such as the superoxide radical anion, hydroxyl radical, hydroperoxyl radical, or hydrogen peroxide.<sup>6</sup> Type 1 photosensitizers can be excited using any wavelength of light; albeit, visible light is preferred for clinical use. In the type 2 process, the energy of the excited photosensitizer is first transferred to the oxygen to generate reactive singlet oxygen that causes cell death. The type 2 process requires the light of wavelength 600–700 nm (red light) range for efficient generation of singlet oxygen.

In the previous paper<sup>7</sup> on type 1 photosensitizers, we reported that sulfenamides, which contain a labile S–N bond, underwent photofragmentation to produce S- and N-centered radicals, and that the N-centered radicals formed initially upon photofragmentation could either induce cell death directly or, in the presence of oxygen, form stable nitroxyl radicals along with ROS. It has been shown that aromatic nitroxyl radicals can intercalate into DNA and cause single strand breakage.<sup>8</sup> Interestingly, aliphatic nitroxyl radicals, in contrast, are known to be cell protectors against oxidative stress.<sup>9</sup> The results of this study on sulfenamides,<sup>7</sup> and previous ESR and laser flash

photolysis studies on diarylamines,<sup>10–16</sup> prompted us to investigate whether diarylamines by themselves would be useful as a type 1 phototherapeutic agent. Accordingly, in this paper, we report the cell viability and ESR results of various diarylamines 1–12 exposed to UV-A/UV-B light (Figure 1).

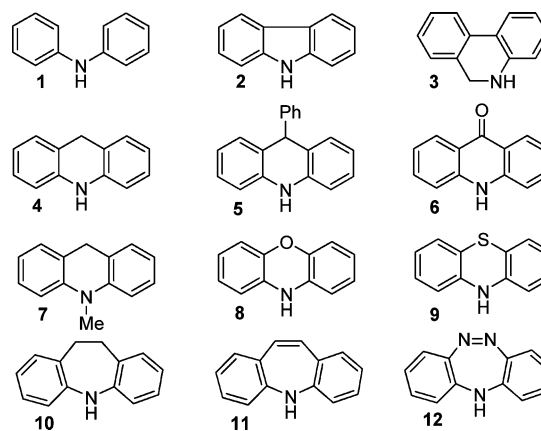


Figure 1. Structures of Diarylamines.

Interestingly, the phototoxicity of amitriptyline, a tertiary diarylamine akin to 7, is due to the effect of superoxide anion,<sup>17</sup> which can be postulated to have been generated via the type 1 process. The diarylamines 1–4, 6, 8, 9, 10, and 11 were purchased from commercial sources. The amines 5, 7, and 12 were prepared by published methods.<sup>18–20</sup>

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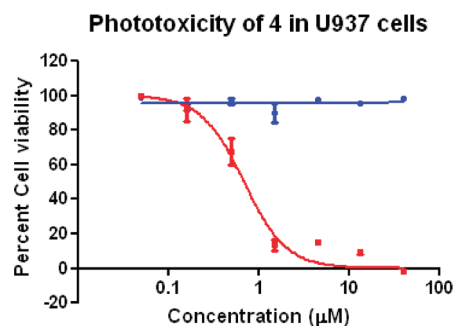
The cancer cell viability assays with the diarylamines above in the absence and presence of light were carried out by the same procedure as described in our previous paper.<sup>7</sup> The compounds were dissolved in DMSO (8 mM) and diluted with the cell culture media such that the amount of the DMSO exposed to the cells remained below 0.5% (64 mM). The IC<sub>50</sub> values (50% decrease in cell viability after exposure to light for 20 min) are given in Table 1.

**Table 1. Cell Viability (IC<sub>50</sub>) of Diarylamines**

| photosensitizer | IC <sub>50</sub> (μM) <sup>a</sup> |
|-----------------|------------------------------------|
| 1               | N.D. <sup>b</sup>                  |
| 2               | 5.56 ± 1.15                        |
| 3               | N.D. <sup>b</sup>                  |
| 4               | 0.68 ± 0.11                        |
| 5               | 0.17 ± 0.01                        |
| 6               | N.D. <sup>b</sup>                  |
| 7               | N.D. <sup>b</sup>                  |
| 8               | 14.5 ± 1.54                        |
| 9               | 1.39 ± 0.11                        |
| 10              | N.D. <sup>b</sup>                  |
| 11              | 2.58 ± 1.14                        |
| 12              | N.D. <sup>b</sup>                  |

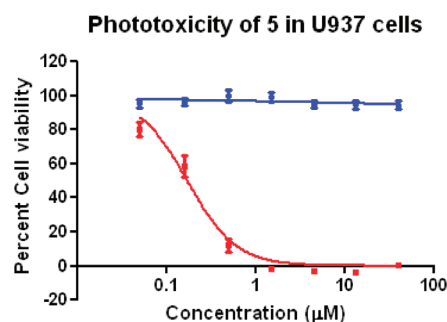
<sup>a</sup>Average of at least three independent runs. <sup>b</sup>Not determined due to the lack of difference between dark-toxicity and phototoxicity in the entire concentration range.

All of the active diarylamines displayed dose-dependent and light exposure time dependent decrease in cell viability. It should be noted that DMSO itself exhibited cytotoxicity only at high concentration and at long exposure time to light.<sup>7</sup> Representative cell viability dose–response graphs for the three most active diarylamines 4, 5, and 11 are shown in Figures 2–4. Diphenylamine (1) is inactive under the

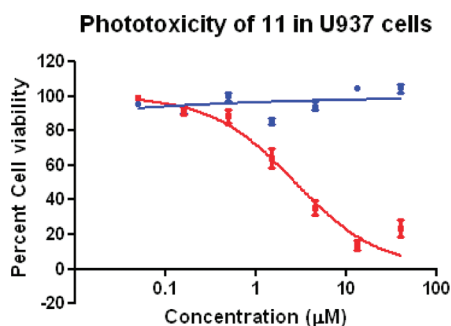


**Figure 2.** Cell viability graph of acridan (4): blue, no light exposure; red, 20 min light exposure.

photolytic conditions employed herein. Connecting the two phenyl groups with a single bond (compound 2) resulted in a moderately active compound with an IC<sub>50</sub> of about 5 μM. Insertion of a methylene bridge between the nitrogen and the phenyl ring (compound 3) completely abrogated the activity. In sharp contrast, insertion of a methylene bridge between the two phenyl rings (acridan, 4) resulted in a very potent compound with an IC<sub>50</sub> of about 0.68 μM. Introduction of the phenyl group in the 9-position in acridan (compound 5) resulted in the most potent compound with IC<sub>50</sub> of 0.17 μM. As would be expected, introduction of a carbonyl group at the 9-position (compound 6) abrogated the activity due to the

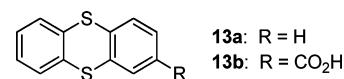


**Figure 3.** Cell viability graph of 9-phenylacridan (5): blue, no light exposure; red, 20 min light exposure.



**Figure 4.** Cell viability graph of dibenzazepine 11: blue, no light exposure; red, 20 min light exposure.

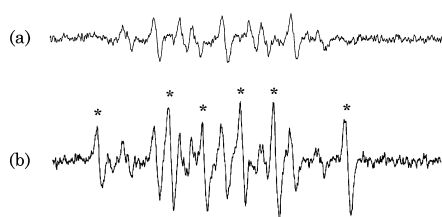
deactivating effect of the carbonyl group toward free radical formation.<sup>21</sup> Likewise, N-methylation of acridan (compound 7) also obviated the activity, clearly indicating the requirement of the NH group for activity. Replacement of the methylene group in acridan with oxygen or sulfur produced varied results. Phenoxazine (8) is weakly active, whereas phenothiazine (9) is highly active. Indeed, the phenothiazine is one of the three most potent compounds, with an IC<sub>50</sub> of about 1.39 μM. Replacement of the NH group in phenothiazine with a sulfur atom (compound 13) completely abrogated the activity, confirming that the nitrogen-centered radical is indeed required for activity. This inactivity is consistent with the fact that no radicals could be detected by ESR with 13b or with the parent



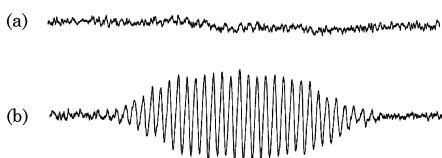
thianthrene (13a).<sup>22</sup> Insertion of an ethylene bridge between the two phenyl rings (compound 10) resulted in inactive compound. In contrast, insertion of a vinylene bridge (compound 11) produced a very active compound with an IC<sub>50</sub> of about 2.58 μM. Surprisingly, introduction of an azo bridge (compound 12) completely abrogated the activity.

All of the diarylamines generated copious free radicals upon photoexcitation, as confirmed by ESR spectroscopy. The properties, lifetimes, and further transformations of the initially formed N-centered radical are obviously dependent on the structure of the corresponding diarylamine from which they had originated. Because most of the free radicals are short-lived, a spin trapping agent, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), was used to trap radical species in the ESR studies for some of the compounds. The DMPO spin adducts are relatively stable nitroxides with unique ESR spin parameters and spectral patterns depending on the type of free radicals

added to the carbon atom at the 2-position (i.e., the  $\beta$ -carbon) in DMPO.<sup>23,24</sup> However, some of the amines, such as **1**, **8**, **9**, and **10**, have been shown to produce stable nitroxyl radicals or radical cations<sup>10,11</sup> that would not require spin trapping. For illustrative purposes, representative ESR spectra of two of the active compounds, viz., acridan (**4**) and the azepine **11**, are shown in Figures 5 and 6, respectively. It should be noted that



**Figure 5.** ESR spectrum of photolyzed acridan (**4**) with DMPO in benzene solution (deoxygenated with nitrogen gas for 5 min): (a) before irradiation; (b) after irradiation for 2 min. The starred peaks are DMPO/acridan spin adducts ( $g = 2.0041$ ) with the following splitting parameters:  $a_{N\sigma} = 1.423$ ;  $a_{H\beta} = 2.111$  mT.

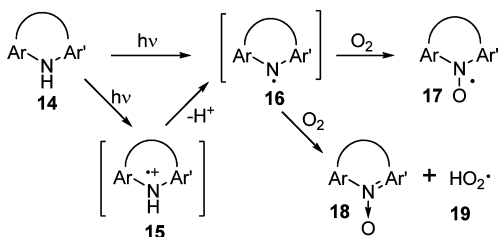


**Figure 6.** ESR spectrum of 5H-dibenz[*b,f*]azepine (**11**) without DMPO in benzene solution: (a) sample without deoxygenation; (b) sample purged with nitrogen for 5-min: nitrogen-centered radical ( $g = 2.0015$ ) with the following fitting parameters:  $a_N = 0.84$ ,  $a_H(2) = 0.42$ ,  $a_H(4) = 0.22$ ,  $a_H(4) = 0.11$  mT; the numbers in the parentheses refer to the number of equivalent protons.

the ESR spectrum of photolyzed acridan (**4**) was obtained in the presence of DMPO but that of the photolyzed azepine **11** was determined without DMPO because the lifetime of the radical ( $t_{1/2} = 20$  s) from the photolysis of **11** was sufficiently long. The complete discussion of the ESR measurement and spectral analyses of the diarylamines **1**–**12** will be published separately.

As reported previously,<sup>11,16</sup> the laser flash photolysis and ESR studies of diarylamines **14** initially yield the aminyl radical cations **15** or aminyl radicals **16** as primary photoproducts depending on the rigidity of the molecule (Scheme 1). For

#### Scheme 1

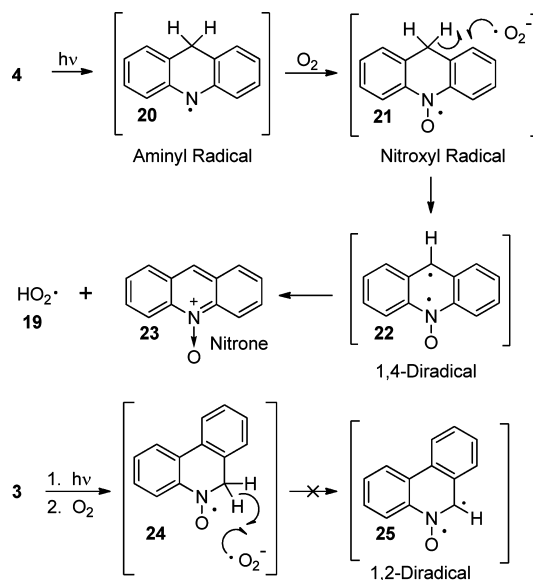


example, the highly rigid carbazole yields only the radical cation **15** initially, whereas phenothiazine (**9**) yields only the aminyl radical **16**. Acridan (**4**) gives predominantly the radical cation **15** along with a small amount of the radical **16**. Nevertheless, the radical cation **15** is eventually converted to the aminyl radical **16** upon deprotonation, and the resulting aminyl radical

**16** may induce tissue damage directly or undergo further reaction with oxygen to form the nitroxyl radical **17** and the hydroperoxyl radical **19**.<sup>10,11,25</sup>

The differences in activity among the diarylamines can be rationalized on the basis of transition states leading to the formation of reactive intermediates. In the case of diphenylamine (**1**) versus carbazole (**2**), the difference could be attributed to better electron delocalization of the N-centered radical (or radical cation) in carbazole compared to diphenylamine, and the ability of planar carbazole to intercalate in the DNA framework. The sharp difference in cell viability between dihydrophenanthridine (**3**) and acridan (**4**) can be explained by the mechanism outlined in Scheme 2. In the case of acridan, the

#### Scheme 2

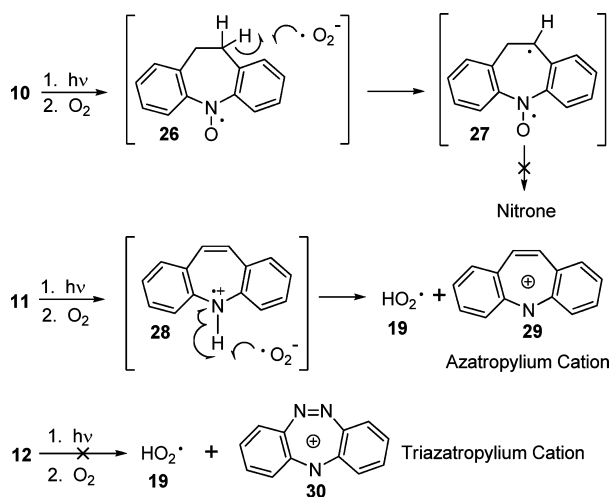


nitroxyl radical may undergo facile hydrogen abstraction by the superoxide anion radical to generate stable acridine nitron (**23**) and hydroperoxyl radical (**19**) via the benzhydryl 1,4-diradical **24**. Indeed, the formation of benzhydryl radical upon photolysis of *N*-methylacridan (**7**) has been reported.<sup>12</sup> The fact that 9-phenylacridan (**5**) is more active than acridan (**4**) provides further support for the mechanism outlined in Schemes 1 and 2. The hydroperoxyl radical **19** is known to be lethal to cells.<sup>8</sup> In contrast, hydrogen abstraction from dihydrophenanthrene nitroxyl radical **24** will yield the benzyl 1,2-diradical **25**, which is less stable than the benzhydryl radical **21**. In addition, the two unpaired electrons adjacent to each other in **25** induce strong electron repulsion, and any rearrangement of the diradical **25** to the corresponding 1,4-diradical will result in loss of aromaticity. Thus, the transition state for the formation of **25** and, hence, the formation of hydroperoxyl radical should be strongly disfavored.

The difference in activity between phenoxazine (**8**) and phenothiazine (**9**) could be attributed to the longer-lived N-centered radical ( $t_{1/2} = 1.0$  s) compared to that of phenoxazine ( $t_{1/2} = 0.3$  s).<sup>10</sup> It was further shown that phenoxazine produced a neutral N-centered radical or radical cation, but not nitroxyl radical. In contrast, phenothiazine produced a mixture of nitroxyl and neutral N-centered radicals, confirming that nitroxyl radicals could cause cell death. The sharp differences in the activities among the tricyclic azepine series of compounds **10**–**12** can be explained by the mechanism outlined in Scheme

3. As in the case of dihydrophenanthrene, although the benzyl 1,4-diradical **27** is more stable than the 1,2-diradical **25**,

Scheme 3



nevertheless, the rearrangement will still result in the loss of aromaticity. The photolysis of dibenzazepine **11** could result in the formation of stable azatropylium cation **29**<sup>7,26,27</sup> and hydroperoxyl radical anion **19** via electron and hydrogen transfer to the oxygen. In the case of triazepine **12**, the transition state leading to the formation of the cation **30** may not be possible due to the strong deactivating effect of the two sp<sup>2</sup> nitrogens of the azo group. Except for diphenylamine and carbazole, the cell viability data of diarylamines parallels the activities of the previously reported sulfenamides<sup>7</sup> derived from the corresponding diarylamines. The diarylamines in this work absorb in the UV-A/UV-B region. To be clinically useful, these diarylamines must not only absorb in the visible region but also display low systemic toxicity. Although anilines have been known to exhibit red cell and liver toxicity,<sup>28,29</sup> interestingly, the diarylamines have been recently shown to play a beneficial role against oxidative stress.<sup>30</sup>

In this preliminary communication we have shown that the secondary diarylamino moiety could be a viable functional group for the design of suitable type 1 photosensitizers. We have demonstrated that the photolysis of diarylamines generates reactive species that destroy the cancer cells. The presence of the hydrogen atom on the nitrogen is a key requirement for activity. This observation is consistent with our previous work with sulfenamides<sup>7</sup> and also with the mechanism outlined in Scheme 1, which supports the formation of nitrogen-centered radicals such as **16** as the initial reactive intermediate. The aminyl radical **16** itself or secondary ROS generated by the reaction of **16** with oxygen could cause cell death, but it remains to be established which of these reactive species is directly responsible for inducing cell death. Further work is in progress to enable the diarylamines to undergo photoexcitation in the visible region, to mitigate systemic toxicity, and to elucidate the precise mechanism of action of these type 1 photosensitizers.

## ■ ASSOCIATED CONTENT

### Supporting Information

In vitro assay procedures and cell viability graphs of compounds **1–3**, **6–10**, **12**, and **13a,b**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

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### Notes

The authors declare no competing financial interest.

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