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Short communication

Phenothiazinium–fluoroquinolone drug conjugates

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ABSTRACT

Synthesis and antibacterial screening of a homologous series of 3-dialkylaminophenothiazinium-7-norfloxacin conjugates was carried out alongside a corresponding series of symmetrical methylene blue derivatives. The norfloxacin conjugates maintained typical methylene blue derivative photoproperties, such as long wavelength absorption, but produced no measurable singlet oxygen in the standard assay and provided no significant increase in the magnitude of photoantibacterial action, this being similar to the methylene blue homologues, although both the conjugates and homologues were considerably more active than methylene blue itself both against *Staphylococcus aureus* and *Escherichia coli*. DNA binding via intercalation was considerably greater for the series of norfloxacin conjugates than for the methylene blue homologues.

1. Introduction

Methylene blue and a number of structurally related phenothiazinium derivatives have been reported to be efficacious photoantimicrobials against a wide range of bacteria, fungi and viruses [1]. Red light absorption by such compounds (620–670 nm) is sufficient to circumvent endogenous absorption by natural chromophores, e.g. haem and melanin, allowing the use of methylene blue derivatives in body fluids and tissues.

Other classes of photosensitiser have been similarly investigated. The porphyrin class is chief among these, mainly due to its majority share in the clinical photodynamic therapy (PDT) of cancer, although this may not supply a sound rationale for antimicrobial discovery. Indeed, whereas the cationic phenothiazinium class exhibits broad-spectrum antibacterial activity, anionic porphyrins used in PDT, such as protoporphyrin IX or haematoporphyrin derivative, are normally only effective against Gram-positive bacteria [2].

To remedy this lack of activity, various groups have investigated the attachment of targeting or adjuvant moieties to anionic porphyrin photosensitisers. Thus, polycationic molecules, such as poly(lysine) and poly(ethyleneimine), have been attached to chlorin e_6 (Ce6), the resulting polycationic side chains having the effect of permeabilising the outer membrane of Gram-negative bacteria allowing photosensitiser ingress and thus photocytotoxicity [3,4]. Anionic porphyrins have also been examined in conjunction with the cationic peptide antibiotic polymyxin, with similar intent [5].

However, such chromophore attachment to peptides and polymers suffers from the fact that there are multiple sites of attachment, in this case between free carboxylic acid residues in the chlorin ring and numerous amine residues in the polymers, often chemically equivalent. Consequently, product mixtures are formed rather than pure material and this may be disadvantageous from the point of view both of structure–activity relationships and of future clinical product registration.

Novel methylene blue derivatives are usually produced by altering the auxochromic amine groups at positions 3 and 7 of the phenothiazinium ring, typically providing homologous series, and both symmetrical and asymmetrical examples have been reported in the literature [6,7]. However, owing to the broad-spectrum activity associated with methylene blue derivatives, the use of biomolecule attachment to the phenothiazinium chromophore has received scant attention, although in the anticancer application attachment of methylene blue via an extra amino group at C-4 to oligonucleotides has been reported [8].

As part of an ongoing photosensitiser discovery programme, it was considered that it might be possible to use small bioactive molecules to aid in photoantibacterial targeting. Given the requirement for single, pure, active species in drug development, the reactive intermediate cation employed in asymmetrical phenothiazinium synthesis was used as a starting point, since this would guarantee a single active site for coupling with amines.

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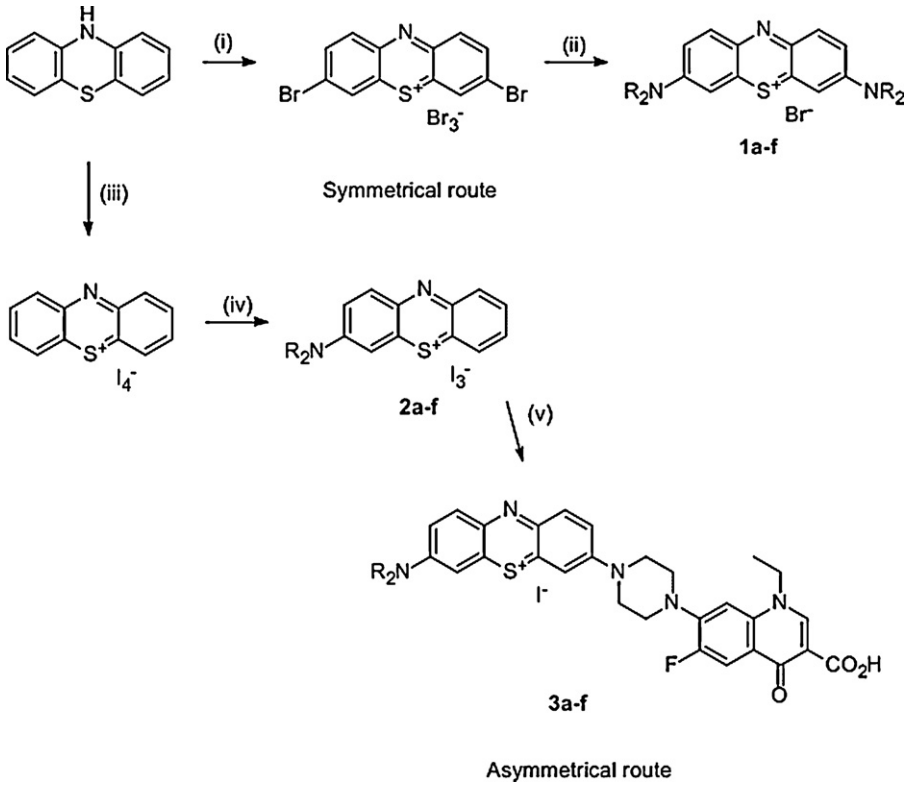

Fig. 1. Synthetic routes to the phenothiazinium homologues. Reagents: (i) bromine in acetic acid; (ii) dialkylamine in dichloromethane; (iii) iodine in dichloromethane; (iv) dialkylamine in methanol; and (v) norfloxacin in methanol.

The fluoroquinolone class of antibacterial agents is one of the most widely used in modern infection control, the presence of a fluorine atom at position 6 of the 4-quinolone ring coupled with amino functionality at the neighbouring position (C-7) providing optimal antibacterial activity [9]. Several front-line fluoroquinolones are substituted in this latter position with a 4-piperazinyl moiety, thus offering ideal potential reactive sites for the current proposition, since this would furnish a dialkylamine equivalent auxochrome in the resulting phenothiazinium derivative (Fig. 1), optimising light absorption in the 660 nm region. A further reason for the use of fluoroquinolones here is their targeting of enzymes closely associated with bacterial DNA [10].

The present study covers the investigation of the antibacterial activity of a series of methylene blue analogues, i.e. having increasing size of dialkylamine group at position 3 and attached at position 7 to the fluoroquinolone norfloxacin via the 4-piperazinyl moiety.

2. Materials and methods

2.1. Materials

The following amines were purchased from Sigma-Aldrich (Gillingham, UK) and were used without further purification: dimethylamine; diethylamine; di-*n*-propylamine; di-*n*-butylamine; di-*n*-pentylamine; and di-*n*-hexylamine. 10*H*-phenothiazine and norfloxacin were also purchased from Sigma-Aldrich. The standard photosensitiser methylene blue (Sigma-Aldrich) was purified by column chromatography on silica gel [gradient elution with 0–10% methanol/dichloromethane (DCM)]. Methanol (spectrophotometric grade), 1-octanol, DCM and iodine were purchased from Fisher Scientific (Leicestershire, UK) and were used without further purification.

2.2. Symmetrical photosensitisers

2.2.1. 3,7-Dibromophenothiazinium tribromide

One gram of phenothiazine (5 mmol) was dissolved in 70 mL of glacial acetic acid, ensuring uniform mixing for approximately 30 min. To this solution, 5 mL of bromine in 40 mL of glacial acetic acid was added rapidly. Then, 200 mL of cold water was quickly added to this mixture with vigorous stirring, resulting in a dark red solution containing a black precipitate. The solution was then filtered through a sintered glass crucible and the resulting solid was washed with water and then with diethyl ether to ensure all bromine was removed. The yield of dark red–brown powder was 2.72 g (81%).

2.2.2. 3,7-Bis(dialkylamino)phenothiazinium bromides (**1b–1f**)

The requisite dialkylamine (15 mmol) in 100 mL of DCM was stirred in a flask followed by the addition of 1.00 g (1.7 mmol) of solid 3,7-dibromophenothiazinium tribromide. The mixture was stirred for approximately 4 h, washed with dilute hydrobromic acid followed by water, the organic layer was separated and dried (Na_2SO_4) and then concentrated under vacuum and the product was precipitated using diethyl ether. Dissolution in DCM and ether precipitation was repeated until spectrophotometric examination of the resulting material gave an absorbance ratio ($A_\lambda : A_{290}$) of 2.2.

2.3. Asymmetrical photosensitisers

Asymmetric analogues were prepared by the initial oxidation of 10*H*-phenothiazine with iodine, but with reaction of the resulting cation with a lowered concentration of amine, furnishing a mono-3-substituted derivative **(2a–f)**. These preparative steps were carried out as in previous work [11]. 3-Substituted intermediates

were further reacted with the second amine, i.e. the 4-piperazinyl unit of norfloxacin, to obtain the asymmetrical product.

2.3.1. Norfloxacin conjugates (**3a–f**)

A solution of the 3-dialkylamino- compound (**2a–f**, 0.75 mmol) in methanol (20 mL) was stirred at room temperature and solid norfloxacin (0.57 g, 1.8 mmol) was added in several portions over 10 min. Progress of the reaction was monitored by thin layer chromatography [11] as described above. The mixture was stirred for approximately 5–6 h at room temperature. The solvent was then removed from the reaction mixture in vacuo and the residual black solid was extracted with DCM. The resulting solution was chromatographed on silica with a gradient MeOH/DCM eluent (0–10% v/v) and the blue fraction was concentrated in vacuo before precipitation using diethyl ether.

Solutions (100 μM) of phenothiaziniums and conjugates were made up in distilled water for use in the bacterial screen. All spectrophotometric measurements were carried out on a Hewlett Packard 8452A diode array spectrophotometer (Agilent Technologies, Waldbronn, Germany). The photosensitisers were found to follow Beer's law in the concentration range of 10^{-5}–10^{-7} mol/L.

2.4. Antibacterial screening

The photobactericidal activities of the norfloxacin conjugates and the methylene blue homologues in addition to those of the known photosensitiser methylene blue and norfloxacin itself were measured against a Gram-positive organism (*Staphylococcus aureus* NCTC 6571) and a Gram-negative organism (*Escherichia coli* NCTC 10418). Both strains were grown in Mueller–Hinton broth and then diluted to a concentration of 10^6 colony-forming units/mL. Aliquots of the strains were treated in microtitre trays with various concentrations of photosensitiser ranging from 100 μM to 3 μM, with zero photosensitiser concentration in each case used for control purposes. The trays were then either illuminated for 20 min using an array of 126 light-emitting diodes (660 nm) giving a light dose of 6 J/cm^2 or alternatively foil-covered to provide dark controls. From each well showing complete absence of growth of the microorganism (i.e. total bacterial kill), 1 μL was subcultured on nutrient agar using the Miles–Misra method and incubated for 18 h at 37 °C.

Minimum bactericidal concentrations (MBCs) were thus determined as the lowest concentration of each photosensitiser giving no bacterial growth. Each test was repeated ($n = 6$) to ensure an absolute value for the cited MBC. Owing to the absolute nature of the assay, i.e. the cut-off point was taken as a complete absence of growth rather than fractional kill, no statistical treatment of the resulting data was applied.

2.5. DNA intercalation

Following previous work [12], a DNA solution was made with 0.75 mg of DNA sodium salt type XIV from herring testes (Aldrich, St Louis, MO) dissolved in 10 mL of distilled water. Using the extinction coefficient (ε) for DNA of 6600 M^{-1} cm^{-1}, the concentration of the DNA solution was found to be 100 μM.

Distilled water solutions of the candidate photosensitisers (100 μM) were made up and λ_{max} values were measured spectrophotometrically.

For each of the candidates, 8 mL of 100 μM DNA solution was mixed in a test tube with 1 mL of the 100 μM photosensitiser solution. The λ_{max} of the resulting mixture was recorded and compared with that of the original photosensitiser solution. Both methylene blue and norfloxacin were included as comparators.

2.6. Singlet oxygen production and lipophilicity (log P)

These properties were measured spectrophotometrically, as recounted in previous work [11].

3. Results

The present study represents the first report of the synthesis of phenothiazinium–drug conjugates. As expected, reaction between the monosubstituted 3-dialkylaminophenothiazinium intermediate compounds and norfloxacin was straightforward owing to the nucleophilic nature of the 4-piperazinyl moiety, providing deep-blue-coloured reaction mixtures (Fig. 1). Products required column chromatography for purification but yields were in line with previous work involving the synthesis of asymmetrical phenothiazinium derivatives [11]. In the same way, the absorption maxima of the conjugates were similar to those of 3-dialkylamino-7-heterocyclophenothiazinium derivatives reported by Gorman et al. [7], since any electronic effects due to the fluoroquinolone moiety are not transmissible to *N*-4 of the piperazinyl subunit (Table 1).

Previously, *N*-4-piperazinyl derivatives of norfloxacin have been synthesised as novel but conventional antibacterial agents using a range of substituents, including heteroaromatics, several of which exhibited increased activity against Gram-negative bacteria including *E. coli* [13]. Simple 4-*N*-alkyl derivatives have also been shown to have increased activity against conventional resistant bacteria, again including *E. coli* and *S. aureus* [14].

Series of symmetrical methylene blue derivatives (**1a–f**; Fig. 1), i.e. having identical dialkylamine groups in positions 3 and 7 of the phenothiazinium ring, have been reported previously by different groups [7,11] and exhibited the expected increase in

Table 1
Analytical data for the derivatives.

Derivative	R^3	R^7	X^-	*m/z*[a]		% Yield	λ_{max} (nm)[b]	Log ε_{max}[b]
				Calculated	Observed			
1a	NMe_2	NMe_2	Cl^-	–	–	–	657	4.88
1b	NEt_2	NEt_2	Br^-	340.18	340.18	23	664	4.87
1c	$NPr^n{}_2$	$NPr^n{}_2$	Br^-	396.25	396.24	28	669	4.75
1d	$NBu^n{}_2$	$NBu^n{}_2$	Br^-	452.31	452.33	30	671	4.84
1e	$NPe^n{}_2$	$NPe^n{}_2$	Br^-	508.37	508.32	21	672	4.69
1f	$NHx^n{}_2$	$NHx^n{}_2$	Br^-	564.43	564.44	18	673	4.55
3a	NMe_2	Norfloxacin	I^-	558.20	558.15	24	658	4.61
3b	NEt_2	Norfloxacin	I^-	586.23	586.05	25	662	4.66
3c	$NPr^n{}_2$	Norfloxacin	I^-	614.26	614.17	30	665	4.77
3d	$NBu^n{}_2$	Norfloxacin	I^-	642.29	642.09	37	665	4.81
3e	$NPe^n{}_2$	Norfloxacin	I^-	670.32	670.27	34	664	4.65
3f	$NHx^n{}_2$	Norfloxacin	I^-	698.35	698.15	33	665	4.69

[a] Molecular ion mass measured by inductively coupled plasma mass spectrometry.
[b] Measured in MeOH.

Table 2
Antibacterial and related data for the derivatives.

Compound	MBC (μM)				Rel.$^1O_2$[a]	Log *P*	DNA shift (nm)
	Staphylococcus aureus		*Escherichia coli*				
	Light	Dark	Light	Dark			
Norfloxacin	100	100	25	25	[b]	+0.1	0
1a	25	100	25	50	1.00	−0.1	+4
1b	6.3	100	6.3	100	0.55	+0.8	+3
1c	3.1	50	6.3	100	0.59	+1.1	+1
1d	3.1	50	3.1	100	0.61	+1.3	+2
1e	3.1	100	3.1	100	0.26	+1.6	+5
1f	3.1	100	3.1	100	0.21	+1.8	+6
3a	6.3	100	6.3	100	[b]	−0.1	+11
3b	3.1	25	3.1	100	[b]	+0.2	+10
3c	3.1	100	3.1	100	[b]	+0.2	+12
3d	12.5	100	12.5	100	[b]	+0.4	+11
3e	12.5	100	12.5	100	[b]	+0.5	+10
3f	12.5	50	12.5	50	[b]	+0.4	+4

MBC, minimum bactericidal concentration.
[a] Yield of singlet oxygen relative to that of methylene blue.
[b] $^1O_2$ measurement ≤2% that of methylene blue.

log *P* and λ_{max} with auxochrome size along the homologous series.

4. Discussion

Although it had been assumed that the presence of the inert piperazinyl linker between the phenothiazinium and fluoroquinolone ring systems would prevent intramolecular interference with singlet oxygen generation by the former under red light conditions, this was not the case. In vitro singlet oxygen yields for the conjugates were, in fact, too low to measure using the standard spectrophotometric assay employed, unlike previous asymmetrical examples (Table 2). However, it is again noted that such tests are not necessarily of great utility as performance indicators for photoantimicrobials owing to the potential influence of local electronic effects in the cellular milieu, not replicated in photofading tests. It should also be recalled that the cytotoxic agents produced during photodynamic antimicrobial chemotherapy (PACT) are very short-lived, e.g. singlet oxygen has a half-life of less than a microsecond before decaying to ground state molecular oxygen [1].

The loss of in vitro singlet oxygen production in the conjugates may be due to molecular aggregation at the concentrations used in the test. The aqueous spectra of the conjugates in the 600–700 nm region were similar in shape to those of the symmetrical derivatives (**1a–f**), i.e. broadened with a small shoulder around 630 nm. However, whilst this aggregation was not deleterious for the symmetrical species, it is possible that in the conjugates this would allow the close proximity of neighbouring phenothiazinium and quinolone chromophores, thus providing a deactivation route. The photobactericidal effects observed (see below) are not thought to develop from such aggregates.

In terms of nucleic acid interaction, it was noticeable that, whilst the symmetrical series of methylene blue derivatives exhibited similar bathochromic shifts to that of methylene blue itself on in vitro mixing with DNA [15], the conjugates exhibited much greater shifts (typically >10 nm) (Table 2). The magnitude of these shifts was more akin to that reported for Taylor's blue (1,9-dimethyl methylene blue), understood to be a much stronger intercalator [15]. The bathochromic shifts observed in the red region of the spectrum underline the hypothesis that intercalation involved the phenothiazinium chromophore, rather than the fluoroquinolone moiety, as no shifting of the norfloxacin λ_{max} (324 nm) was observed.

It should be noted at the outset that testing of the photosensitisers against bacteria carried out in this work was aimed at a clinically realistic approach. Thus, the candidate photosensitisers were mixed with the bacterial suspensions and illuminated almost immediately with red light for 20 min. Antibacterial screening of conventional agents usually entails a significant incubation time in order for the drug to be taken up by the bacterial cells, typically 18 h. Consequently, under the same conditions as those used for the phenothiazinium derivatives, norfloxacin was much less effective than might normally be expected. In addition, although the fluoroquinolones are also known to be photosensitisers, the use of red light for activation, rather than ultraviolet, elicited photoactivation only of the phenothiazinium chromophores involved.

All of the conjugates and symmetrical analogues of methylene blue exhibited greater efficacy in both organisms than either methylene blue itself or norfloxacin (Table 2). The pattern of increasing activity with alkyl group size among the symmetrical derivatives was not repeated in the conjugates, the dibutyl-dihexyl analogues (**3d–f**) among the latter exhibiting much lower efficacies. Whilst these activity profiles may correlate with lipophilicity for the symmetrical derivatives, this was not the case with the conjugates, since little variation in log *P* was observed across the series, presumably due to the highly hydrophilic character of the norfloxacin moiety.

Dark toxicity between both sets of derivatives was found to be low, most derivatives being toxic at the highest concentrations used (100 μM) or above, in line with methylene blue (Table 2). This suggests a lack of essential targeting and does not support a specific DNA-localising hypothesis. Bacterial DNA photodamage has been reported for methylene blue itself, but it has been suggested that the initial activity occurs at the cell exterior [1]. In addition, the relatively amphiphilic nature of the norfloxacin derivatives here ($-0.1 < \log P < +0.5$) suggests some difficulty in reaching the interior of mammalian cell nuclei, since such localisation has been not reported for the symmetrical derivatives [7].

An increasing number of phenothiazinium derivatives is appearing in the literature as potential biological photosensitisers. As far as we are aware, the present study is the first time that conjugation of the phenothiazinium chromophore to a standard drug has been reported.

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