Title: Fluoroquinolone photodegradation influences the specific basophil activation.

Short title: Quinolone photodegradation affects basophil activation

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Allergy, basophil activation, IgE, quinolones, photodegradation, hapten-protein conjugates.

ABSTRACT

Fluoroquinolones (FQs) are photoreactive drugs, but it is not known whether laboratory light exposure can influence the induction of photoproducts and modify in vitro test results. The basophil activation test (BAT) has proven to be useful for evaluating IgE-mediated hypersensitivity to FQs, with a higher percentage of positive responders with ciprofloxacin (CIP) than with moxifloxacin (MOX). We studied the effect of laboratory light on CIP and MOX degradation, and drug-protein conjugate formation, and its influence on the BAT for evaluating IgE-mediated hypersensitivity to FQs. The results showed an important decrease in the fluorescence emission intensity under light compared to dark conditions for MOX, and that BAT positivity was lower in light (17.9%) than in dark (35.7%). No changes were found for CIP in either fluorescence emission intensity or BAT results (46.4% in both conditions). We can conclude that light exposure is a critical factor in the BAT results when photolabile drugs like moxifloxacin are used. Therefore, light is important when interpreting *in vitro* results.

INTRODUCTION

Quinolones have been used for more than thirty years to treat a wide range of infections. Ultraviolet radiation induces their photodegradation, which is modulated by the nature and position of the substituents attached to the quinolone skeleton [1,2]. For example, the presence of a halogen, as in fluoroquinolones (FQs), seems to be associated with a higher phototoxic potential [3,4]. Photodecomposition may involve a variety of photochemical processes, such as generation of singlet oxygen, production of superoxide, defluorination, decarboxylation at C-3 or oxidation of the amino group at C-7 [1,2,4].

Generally FQs are well tolerated [5], although the last decade has witnessed an increasing number of immediate hypersensitivity reactions (IHR) induced by FQs, with urticaria and anaphylaxis the most frequently reported reactions [6-8]. These observations, especially the occurrence of more severe reactions, have been associated with the introduction of moxifloxacin (MOX) for therapeutic use [6]. In fact, in a group of patients diagnosed with IHR to FQs, MOX was involved in more than 60% of the cases with more severe reactions, followed by ciprofloxacin (CIP) in 30% and, to a much lower extent, levofloxacin [8].

Evidence supporting an IgE mechanism for IHR has been provided by the detection of specific antibodies, by both immunoassay and basophil activation tests (BAT), with different patterns of cross-reactivity among FQs [7,8]. Despite these findings, the true nature of the haptenic substructure (from the parent drug or its metabolites) recognized by the immune system remains unknown. The BAT is an adequate model for studying IgE-mediated reactions to FQs because, in addition to sensitized basophils, it enables study of the hapten, both free and protein bound, as well as its metabolites.

Previous evidence from well-validated CIP and MOX IHR cases suggests that basophil activation occurs to CIP more often than to MOX, even in those cases where MOX was the culprit drug [8]. Because each FQ exhibits chemical differences, our hypothesis was that they may behave differently upon light exposure, which may influence the formation of drug-protein conjugates and therefore interfere with the basophil activation. To test this hypothesis we investigated how light exposure can affect the BAT results in patients with IgE-mediated hypersensitivity reactions and controls with good tolerance to these FQs.

MATERIAL AND METHODS

The stability of the FQs when exposed to laboratory light was checked by spectrophotometric and fluorometric measurements in an aqueous solution and in

supernatants obtained from the BAT. These supernatants were divided into high molecular weight fractions (>3000Da), containing the drug bound to the serum proteins, and low molecular weight fractions (<3000Da), with the free drug or its metabolites, before analysis.

BAT was done as described [8] under light and dark conditions with whole blood from patients with confirmed immediate hypersensitivity to CIP (N=15) or MOX (N=13) and quinolone tolerant controls (N=20). Results were considered as positive when the stimulation index (SI), calculated as the ratio between the percentage of degranulated basophils with the haptens and the spontaneous basophil activation, was greater than 3. Detailed information about the photochemical and biological studies is available in the Supplemental Material.

RESULTS

Photostability of ciprofloxacin and moxifloxacin

Absorption spectra of CIP and MOX showed a wide wavelength band reaching up to 400 nm (Figure S1A Supplementary Material). Emission studies were performed by excitation at 320 nm and 337 nm for CIP and MOX, respectively, displaying different emission bands centered at 420 nm for CIP and 460 nm for MOX. Neither CIP nor MOX exhibited significant spectroscopic changes under light or dark conditions in aqueous solution, indicating a low photodegradation (Figure S1B).

We then analyzed the effect of laboratory light on FQ degradation and on their capability to form drug-protein conjugates in whole blood, the medium used in BAT. The emission data of the low and high molecular weight fractions showed few, if any differences, in the fluorescence intensity for CIP under light or dark conditions, either in free or protein fractions (Figure 1). However, remarkable differences were observed for MOX in both fractions, with an important decrease in the fluorescence emission intensity upon light exposure, indicating drug photodegradation.

BAT results

Twenty-eight patients with confirmed IHR to CIP and MOX and 20 controls with confirmed good tolerance to FQs were evaluated (Table 1 and Supplemental Material). Figure 2 shows the dose response curve with four different concentrations of CIP and MOX, in light and dark in 16 allergic patients and 15 controls. The optimal concentrations were found to be 0.2 and 2 mg/mL for both drugs, and these concentrations were used throughout the study.

Table 1 shows the results of the BAT for CIP and MOX in light and dark for the individual cases, with positive cases shown shaded. For CIP, BAT was positive in 13 cases (46.4%) under light conditions and in 13 cases (46.4%) under dark conditions.

For MOX, BAT was positive in 5 (17.9%) under light conditions and in 10 (35.7%) under dark conditions. Results were positive to either of the two FQs in 13 cases (46.4%) under light conditions and in 16 cases (57.1%) under dark conditions. Figure S2 shows the dot-plot in light and dark of two representative cases.

Analysis of the results depending on the FQ involved in the reaction showed that in those cases where CIP was the culprit drug (N=15), BAT was positive to CIP in 5 (33.33%) in light conditions and 6 (40%) in dark; and to MOX in 6 (40%) in light and 4 (26.66%) in dark; and to either of the two quinolones in 5 (33.33%) in light and 8 (53.33%) in dark conditions.

In those cases where MOX was the culprit drug (N=13), BAT was positive to CIP in 8 (61.53%) in light and 7 (53.84%) in dark; and to MOX in 2 (15.38%) in light and 6 (46.15%) in dark; and to either of the two FQs in 8 (61.53%) in both light and dark conditions.

In controls (N=20), under light conditions, BAT was positive in 2 cases to CIP, 1 case to MOX, and 2 cases to at least one FQ; and in dark, BAT was positive in 1 case to CIP, 2 cases to MOX, and 2 cases to at least one FQ. As a result, the specificity was 90% in both light and dark.

DISCUSSION

The presence of IgE antibodies to FQs has been demonstrated by immunoassays, including inhibition studies, although they do not enable us to determine the hapten determinant involved [7,8]. Recently, BAT has proven to be a useful tool for evaluating IgE responses to these drugs, though the results seem to depend on the FQ used in the test, and are lower with MOX [8]. This, together with the fact that in recent years there has been an increase in the number of MOX reactions, in most cases severe, [6,8] make it important to analyze in depth the factors influencing these different behaviors. Based on the photolability of FQs [1-3,9] our hypothesis was that the differences found in the BAT assay between the FQs may be explained by changes induced under light exposure during the *in vitro* test procedure, which influences FQ degradation differently, producing a lower amount of drug-protein conjugates.

The results obtained from fluorescence emission studies suggest that during BAT both FQs are able to bind to blood proteins, although free drug also remains. The data show an important photodegradation under laboratory light conditions, especially for MOX; as a consequence, lower drug-protein conjugates are also obtained. These results could be explained in terms of photostability since both FQs, although they present the same basic structure, show a different photochemical behavior due to different substituents [1,2]. Even though the presence of a fluorine atom in C-6 makes

both drugs somewhat photoreactive, the degree to which the molecules are photolabile is modulated by substituents and is directly related to the electronegativity of the substituent at C-8 [3,4]. When we analyzed the photostability in aqueous solution we observed a similar behavior for both FQs. However, important differences, with a high MOX degradation, were found when the same experiments were done in whole blood, mimicking the BAT conditions. This may be explained by the fact that the photochemical behavior also depends on the characteristics of the medium, particularly in biological environments [1,2]. Thus, in this study the complexity of the blood samples showed the unexpected facet of the reactivity of these FQs, which makes it difficult to formulate a hypothesis about the mechanisms involved.

These data explain why BAT positivity under light exposure was lower with MOX (17.9%) than with CIP (46.4%), with no patients being positive solely to MOX, as previously reported [8]. However, when the BAT results were analyzed under dark conditions, there was an increase in the number of positive cases to at least one FQ, from 46.4% to 57.1%.

The results obtained in BAT with the lower response observed with MOX in light (17.9%) compared to dark (35.7%) and no changes in the positivity for CIP (46.4%) correlate with the different photobehavior observed in these FQs, finding degradation after light stimulation only in MOX. Thus, in order to improve the sensitivity of BAT with MOX, this assay should be carried out under dark conditions to avoid drug photodegradation and possible misleading results.

Analysis of the results depending on the culprit drug showed that in patients in whom MOX was responsible, in light conditions only 15.38% were BAT positive to this drug while 61.53% were positive to CIP. Thus, although the culprit drug was MOX, most cases were positive to CIP, as in the study by Aranda [8]. This phenomenon was not detected when CIP was the culprit drug. The reason for this was that all the positive cases to MOX were also positive to CIP, indicating that although the reaction was induced by MOX, IgE recognition was in part directed to CIP. Similar results have been published for patients with IHR to amoxicillin or amoxicillin-clavulanic acid where IgE mainly recognized benzylpenicillin [10,11]. This seems to indicate that IgE antibodies are related to the drug first exposed to (benzylpenicillin and ciprofloxacin), even if no previous reaction occurred, thus reflecting an anamnestic immune response [10,11]. The occurrence of this phenomenon is expected to decrease over time as MOX consumption increases compared to CIP, as demonstrated with betalactams where benzylpenicillin is no longer the structure most often recognized [11,12].

Finally, a question remains as to whether the lower sensitivity for MOX found in BAT could also affect other *in vitro* tests, such as the radioimmunoassay. This may be

the case since a lower sensitivity was found with sepharose-RIA to MOX (18%) compared to that achieved with CIP (21%) in the study by Aranda [8], although further research is needed to analyze whether this phenomenon may influence other *in vivo* or *in vitro* tests.

Summarizing, the data reported here suggest that MOX is sensitive to ambient laboratory light present during the performance of an in vitro assay such as BAT, producing higher drug photodegradation and, as a consequence, lower amounts of drug-protein conjugates. This shows that light exposure is a critical factor in the results of the BAT when photolabile drugs are used and it is important to bear this in mind when interpreting *in vitro* results.

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257 **Table 1**. Clinical characteristics and basophil activation test results of the patients

Pat.	Age	Reaction	Time* (months)	Drug	%CD63 CIP		%CD63 MOX		SI CIP		SI MOX	
					Light	Dark	Light	Dark	Light	Dark	Light	Dark
1	60	Urticaria	2	MOX	47.27	53.5	15.57	29.79	8.16	9.24	2.69	5.15
2	74	Anaphylactic Shock	12	MOX	5.72	41.9	13.96	3.9	0.73	5.33	1.78	0.50
3	67	Urticaria	3	MOX	35.39	5.95	2.91	3.83	8.87	1.49	0.73	0.96
4	58	Anaphylaxis	12	MOX	19.54	1.95	4.07	18.82	3.32	0.33	0.93	3.20
5	67	Anaphylactic Shock	2	MOX	40.47	42.86	23.96	18.77	6.30	6.68	3.73	2.92
6	24	Anaphylaxis	3	MOX	26.27	59.55	12.94	99.98	4.99	8.74	2.42	18.72
7	31	Anaphylaxis	12	MOX	23.3	40.94	6.11	48.21	4.13	6.27	1.08	8.55
8	44	Anaphylactic Shock	7	MOX	18.75	3.81	4.39	9.85	2.83	0.58	0.71	1.49
9	65	Anaphylaxis	3	MOX	7.83	12.4	10.58	10.05	1.87	2.97	2.53	2.41
10	59	Urticaria	14	MOX	24.12	20.59	5.96	21.55	4.04	3.45	1	3.61
11	18	Anaphylaxis	1	MOX	5.05	0	3.63	0.56	0.71	0	0.51	0.08
12	45	Anaphylaxis	1	MOX	1.41	0.70	0.75	1.27	0.30	0.15	0.16	0.27
13	63	Anaphylaxis	4	MOX	31.27	23.42	22.92	15.62	5.50	4.12	4.03	3.08
14	41	Anaphylaxis	3	CIP	25.45	11.61	21.23	16.17	2.99	1.37	2.50	1.90
15	39	Anaphylaxis	12	CIP	30.26	51.19	10.95	9.86	5.31	8.98	1.92	1.73
16	16	Anaphylaxis	2	CIP	1.56	23.81	0.79	3.18	0.32	4.94	0.16	0.66
17	58	Anaphylaxis	10	CIP	3.19	15.24	4.97	24.64	0.59	2.83	0.92	4.58
18	16	Anaphylaxis	10	CIP	9.72	39.82	9.68	75.51	1.72	7.06	1.72	13.39
19	39	Anaphylaxis	3	CIP	23.53	3.23	33.33	24.29	3.95	0.54	5.59	4.08
20	53	Anaphylaxis	1	CIP	17.06	2.67	18.6	1.86	3.12	0.49	3.40	0.34
21	23	Anaphylaxis	16	CIP	34.88	19.31	19.43	20.43	6.82	3.78	3.80	4
22	37	Anaphylaxis	10	CIP	7.74	4.01	10.9	4.63	1.37	0.71	1.93	0.82
23	41	Urticaria	1	CIP	2.98	4.12	3.5	8.15	0.63	0.87	0.74	1.72
24	35	Urticaria	12	CIP	12.59	31.92	7.51	6.15	2.23	5.65	1.33	1.09
25	67	Anaphylaxis	3	CIP	11.2	11.19	5.99	6.7	2.33	2.33	1.25	1.39
26	22	Anaphylaxis	5	CIP	4.3	8.87	8.37	9.94	0.83	1.71	1.61	1.92
27	47	Urticaria	12	CIP	6.5	7.75	6.48	3.74	1.22	1.45	1.21	1.34
28	57	Urticaria	3	CIP	18.1	18.46	0.93	1.69	3.20	3.26	0.16	0.37

258 Pat, Patient; SI, Stimulation Index in basophil activation test; CIP, Ciprofloxacin; MOX,
259 Moxifloxacin; * Time between adverse reaction and study. Shaded cells indicate positive SI
260 (greater than 3)

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FIGURES

Figure 1. Mean and standard deviation of emission fluorescence spectra of ciprofloxacin and moxifloxacin in light and dark conditions, obtained from two different fractions, greater and lower than 3000 Da.

Figure 2. Basophil activation test dose response curves for ciprofloxacin and moxifloxacin in 16 patients, 8 with a reaction to MOX and 8 with a reaction to CIP and 15 controls in light and dark conditions. Results are expressed as stimulation index (SI).