Inhibition by New Glucocorticoid Antedrugs [16 α , 17 α -d] Isoxazoline and [16 α , 17 α -d]-3'-Hydroxy- Iminoformyl Isoxazoline Derivatives of Chemotaxis and CCL26, CCL11, IL-8, and RANTES Secretion

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The underlying inflammation present in chronic airway diseases is orchestrated by increased secretion of CC and CXC chemokines that selectively recruit the leukocyte populations into the pulmonary system. Human chemokines, eotaxins (CCL11 and CCL26), RANTES, and interleukin (IL)-8, are dramatically upregulated through G-protein receptors in cell inflammation, including human asthma. In previous studies, a series of new glucocorticoid antedrugs (GCAs) were synthesized as derivatives of isoxazoline and oxime, and their pharmacological properties based on the antedrug concepts were evaluated. Utilizing both human airway epithelium (HAE) and eosinophil (EOS) cell culture models, we carried out studies to test the hypothesis that new GCA cell treatment would ameliorate Th-1/Th-2-driven secretion of these asthmatic biomarkers, eotaxins (CCL11 and CCL26), RANTES, and IL-8 chemokines, that would in turn decrease recruitment, proliferation, and activation of EOS cells. Results demonstrate that isoxazoline and oxime derivatives exhibit concentration-dependent inhibition, and specifically the compound No. 7 decreases significantly the secretion of eotaxins, RANTES, and IL-8 in cytokine-stimulated HAE cells. It was shown that EOS proliferation and activation were reduced considerably, and cell apoptosis occurred when exposed to nonfluorinated isoxazoline derivatives. These results provide evidence that concentration and structural manipulation of GCAs could increase the anti-inflammatory potency in treatment of chronic diseases, including asthma.

Introduction

THE CLINICAL USE of glucocorticoids (GCs) in a wide variety of inflammatory and autoimmune disorders has made them among the most widely prescribed drugs in the world. The anti-inflammatory treatment of using GCs may have wide effects inhibiting the generation of cytokines and chemokines, and inhibiting the development and recruitment of airway eosinophilia (Barnes 1995; Barnes and others 1998b; Lloyd 2002). However, the clinical use of corticosteroids is limited due to a number of serious systemic side effects, some of which are life threatening (Masi and Chrousos 1996; Nishimura and Ikuyama 2000; Alekseev and others 2001; Sambrook and others 2001).

Considerable effort has been exerted to determine the mechanisms of GC activities during inflammation and asthma. GC molecules mainly target intracellular GC receptors (GRs), which are located in the cytosol of airway cells. After infiltrating the cell membrane, GCs bind to the GRs

inactivated by association with multichaperone proteins (Barnes 1996), causing a conformation change that dissociates the GRs from the chaperone proteins. The GRs then dimerize, forming active GC-GR complexes that translocate from the cytosol to the nucleus of the cell, where they bind to specific DNA sequences called glucocorticoid-response elements (GREs) in the promoter region of the GC-regulated genes (Encio and Detera-Wadleigh 1991; Muller and Renkawitz 1991). The GR-GRE association leads directly to transcription activation or repression of the target gene during the inflammatory process. The GC-GR complex can also indirectly interact with transcription factors such as AP-1 or NF- κ B to decrease the transcription of many disease-specific genes, including chemoattractants, cytokines, cytokine receptors, and cell adhesion molecules. This transrepression process involves recruitment of histone deacetylases and modulation of the chromatin structure (Adcock and Ito 2000). The effects of GCs on post-transcriptional mechanisms have also been suggested. GCs induce mRNA destabilization and

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decrease the protein secretion of cytokines interleukin (IL)-1 and IL-6 (Kern and others 1988; Amano and others 1993). GCs can also decrease the expression of the IL-4R receptor during post-transcription, as well as during the translational or post-translational process in response to gene induction (Mozo and others 1998).

Overall, the shortcomings of steroid therapy are largely inherent in their structural features. A considerable amount of research has been conducted to increase the therapeutic index of potent corticosteroids by reducing their systemic side effects. While many new steroids have been obtained by extension of traditional molecular and structural manipulations, new GC molecules with significant structural changes have also been developed (Alekseev and others 2001). Glucocorticoid antedrug (GCAs) are designed compounds that exert desirable effects at the application site, but are rapidly biotransformed to an inactive metabolite by a predictable enzymatic reaction upon entry into the circulation, thus resulting in reduction of adverse systemic effects such as suppressive effect on the hypothalamus-adrenal axis (Lee and Soliman 1982; Lee and Ko 1999).

The common structural feature found in GCAs is the incorporation of a new metabolically labile functionality such as a carboxyester, lactone, or isoxazoline, which is hydrolyzed to an inactive metabolite by enzymes in the circulation (Kimura 1994; Little and others 1999; Procopiou and others 2001; Sawa and others 2002; You and others 2002). Although this concept was developed to synthesize anti-inflammatory steroids, its application has been expanded to other classes of agents. Diversity in therapeutic and structural classes of GCAs has also grown (Bodor 2000; Hwang and others 2000; Ji and others 2000; Khan and others 2005). In accordance with the antedrug concept, our laboratory synthesized fused heterocyclosteroidal antedrug of [16 α , 17 α -d]-3'-hydroxy-iminoformyl isoxazoline and [16 α , 17 α -d] isoxazoline derivatives from the molecular parents prednisolone (PRED), 9- α -fluoroprednisolone (FPRED), and 21-acetyloxy-FPRED FP-21AC (Kwon and others 1995, 2006; Khalil and others 1996; Ko and others 1997). Previously, these compounds showed dose-dependent anti-inflammatory effects in an ear edema bioassay, and displayed no significant suppressive effects on corticosterone levels or thymus weights in the 5-day multiple-dose edema assay (Khalil and others 1996; Ko and others 1997; Park and others 2006).

Chemokines are a superfamily of over 50 small secreted proteins so named for their precise orchestration of leukocyte trafficking in both homeostatic and disease states. Within the recently devised systematic nomenclature, the chemokines are divided into 4 groups based upon the arrangement of their aminoterminal cysteine residues. The largest groups are designated CC and CXC for their adjacent cysteine amino acids or cysteine residues separated by a single amino acid, respectively (Bacon and others 2002). Chemokine effects are mediated by over 20 G protein-coupled receptors also grouped into classes, including CC chemokine receptors (CCRs) that generally bind with the CC chemokines and CXC chemokine receptors that generally limit their binding to CXC ligands (Murphy and others 2002; Pease and Williams 2006). The bioactivities and functions of the chemokines and cytokines extend well beyond their initially identified chemotaxis properties. In addition to their integral roles in both the innate and adaptive arms of the immune system, chemokines and cytokines are implicated in the

pathogenesis of diseases, including rheumatoid arthritis, atherosclerosis, multiple sclerosis, inflammatory diseases of the skin and lung, chronic obstructive pulmonary disease (COPD), allergic rhinitis, and asthma (Charo and Ransohoff 2006). In addition to their specifically defined pathologies, one pulmonary problem common to emphysema, chronic bronchitis, COPD, and asthma is an underlying and complex lower airway inflammation regulated by chemokine and cytokine receptor/ligand signaling. Airway structural or constituent cells that include fibroblasts, myofibroblasts, endothelial cells, smooth muscle cells, and airway epithelial cells are important sources of chemokines that play pivotal roles in the perpetuation of the underlying airway inflammatory responses. At the sites of chronic inflammation, there is selective upregulation of CC chemokines, which then orchestrate recruitment and proinflammatory bioactivities of specific leukocytes. In the case of lung inflammation, for example, asthma, there is a selective recruitment of Th-2 lymphocytes, dendritic cells, basophiles, mast cells, and eosinophils (EOS). Compelling evidence suggests that recruitment is partially in response to increased expression of a subset of CC chemokines that includes eotaxin-1 (CCL11), eotaxin-2 (CCL24), eotaxin-3 (CCL26), RANTES (CCL5), MCP-1 (CCL2), MCP-4 (CCL13), thymus and activation-regulated chemokine (CCL17) and monocyte-derived chemokine (CCL22) (Fan and others 2005), and IL-8 (CXCL8) (Collins and others 1993; Sehmi and others 1993; Villar and others 1993; Warringa and others 1993; Schweizer and others 1994). Following these chemotactic events, bidirectional activating interactions between the emigrating leukocytes and the lung-resident cells engage the self-sustaining proinflammatory cycle (Holgate 2007). Studies have shown a strong correlation between the EOS numbers in bronchoalveolar lavage, endobronchial biopsies, and sputum and hyper-responsiveness of the airways (Green and others 2002). This characteristic accumulation of EOS is directed, in part, by EOS-selective chemokines and their receptors. The most selective chemokines for EOS trafficking and recruitment are the 3 eotaxins, CCL11, CCL24, and CCL26, (Ponath and others 1996b; Phillips and others 2003; Garcia and others 2005), RANTES, and IL-8 (Resnick and Weller 1993).

In this study, we examine the anti-inflammatory inhibition effects of different GCAs on several chemokine biomarkers, including eotaxin-1 (CCL11), eotaxin-3 (CCL26), RANTES (CCL5), and IL-8 (CXCL8), involved potentially in alveolar airway epithelium cell inflammation and EOS cell inflammation and recruitment. The anti-inflammatory potency of GCAs and their concentration-dependent effects are also studied. Additionally, this study focused on cytokine-induced cell proliferation and GCA-induced cell apoptosis in EOS.

Materials and Methods

Chemicals and reagents

All chemicals were purchased from Sigma Company. The 3 GCs, 21-acetyloxy-FPRED, PRED, and FPRED, as molecular parents were purchased from Pharmacia & Upjohn Company. New GCAs were synthesized in this laboratory as previously described (Kwon and others 1995, 2006; Khalil and others 1996; Ko and others 1997) and are presented in Table 1 and Fig. 1. These are 21-acetyloxy-11 β -hydroxy-3,20-dioxo-1,

TABLE 1. NAME AND NOMENCLATURE FOR GLUCOCORTICOID ANTEDRUGS

No.	Compounds	Names
1	P-ISO-21AC	21-acetyloxy-11 β -hydroxy-3,20-dioxo-1,4 pregnadieno [16 α ,17 α -d] isoxazoline
2	FP-ISO-21AC	21-acetyloxy-9 α -fluoro-11 β -hydroxy-3,20-dioxo-1,4pregnadieno [16 α ,17 α -d] isoxazoline
3	P-ISO-ET-21OH	11 β ,21-dihydroxy-3,20-dioxo-3'-(ethoxycarbonyl) isoxazolino [16 α ,17 α -d] pregna-1,4-diene
4	FP-ISO-ET-21OH	11 β ,21-9 α -fluoro-dihydroxy-3,20-dioxo-3'-(ethoxycarbonyl) isoxazolino [16 α ,17 α -d] pregna-1,4-diene
5	P-ISO-ET-21AC	21-acetyloxy-11 β -hydroxy-3,20-dioxo-3'-(ethoxycarbonyl) isoxazolino [16 α ,17 α -d] pregna-1,4-diene
6	FP-ISO-ET-21AC	21-acetyloxy -11 β ,21-dihydroxy-9 α -fluoro-3,20-dioxo-3'-(ethoxycarbonyl) isoxazolino [16 α ,17 α -d] pregna-1,4-diene
7	P-OXIM-21AC	21-acetyloxy-11 β -hydroxy-3,20-dioxo-1,4-pregnadieno [16 α ,17 α -d]-3'-hydroxy-iminoformyl isoxazoline
8	FP-OXIM-21AC	21-acetyloxy-9 α -fluoro-11 β -hydroxy-3,20-dioxo-1,4-pregnadieno [16 α ,17 α -d]-3'-hydroxyiminoformyl isoxazoline
9	P-OXIM-21OH	11 β ,21-dihydroxy-3,20-dioxo-1,4-pregnadieno [16 α ,17 α -d]-3'-hydroxy-iminoformyl isoxazoline
10	FP-OXIM-21OH	11 β ,21-dihydroxy -9 α -fluoro-3,20-dioxo-1,4-pregnadieno [16 α ,17 α -d] 3'-hydroxy-iminoformyl isoxazoline
11	FP-21AC	21-acetyloxy-9 α -fluoro-fluoroprednisolone
12	PRED	Prednisolone
13	FPRED	9- α -fluoroprednisolone

4-pregnadieno [16 α ,17 α -d] isoxazoline (P-ISO-21AC); 21-acetyloxy-9-fluoro-11 β -hydroxy-3,20-dioxo-1,4-pregnadieno [16 α ,17 α -d] isoxazoline (FP-ISO-21AC); 11,21-dihydroxy-3,20-dioxo-3-(ethoxycarbonyl) isoxazolino [16 α ,17 α -d] pregna-1,4-diene (P-ISO-ET-21OH); 11,21-dihydroxy-9-fluoro-3,20-dioxo-3-(ethoxycarbonyl) isoxazolino [16 α ,17 α -d]pregna-1,4-diene(FP-ISO-ET-21OH); 20-acetyloxy-11-hydroxy-3,20-dioxo-3-(ethoxycarbonyl) isoxazolino [16 α ,17 α -d] pregna-1,4-diene (P-ISO-ET-21AC); 20-acetyloxy-9-fluoro-11-hydroxy-3,20-dioxo-3-(ethoxycarbonyl) isoxazolino [16 α ,17 α -d] pregna-1,4-diene (FP-ISO-ET-21AC); 21-acetyloxy-11-hydroxy-3,20-dioxo-1,4-pregnadieno [16 α ,17 α -d] -3'-hydroxyiminoformyl isoxazoline (P-OXIM-21AC); 21-acetyloxy-9-fluoro-11-hydroxy-3,20-dioxo-1,4-pregnadieno[16 α ,17 α -d]-3'-hydroxyiminoformyl isoxazoline (FP-OXIM-21AC); 11 β ,21-dihydroxy-3,20-dioxo-1,4pregnadieno [16 α ,17 α -d] -3'-hydroxyiminoformyl isoxazoline (P-OXIM-21OH); and 11 β ,21-dihydroxy-9-fluoro-3,20-dioxo-1,4-pregnadieno[16 α , 17 α -d]-3' hydroxyiminoformyl isoxazoline (FP-OXIM-21OH). The structures of these compounds are shown in Fig. 1.

Cell culture

Human A549 alveolar type II epithelial-like cells (HAE cells) [American Type Culture Collection (ATCC) CCL-185] were grown in RPMI-1640/F-12K (50:50 vol/vol) supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μ g/mL) in a humidified atmosphere of 5% carbon dioxide at 37°C. Trypsin/EDTA-dispersed cells or phosphate-buffered saline (PBS)/EDTA-dispersed cells were suspended in a fresh medium in flasks or wells at 1×10^5 cells/mL. EOS cells Clone 15 HL-60 (EOS cells) (ATCC CRL-1964) were cultured in RPMI 1640 (Cellgro by Mediatech, Inc.) supplemented with 10% fetal calf serum, 10 ng/mL IL-5 (Atlanta Biologicals), penicillin (100 U/mL), and streptomycin (100 μ g/mL) in a humidified atmosphere as described above. Final differentiation was carried out for 7 days in supplemented RPMI-1640 containing 0.5 mM n-butyric acid (Errahali and others 2009). Viability of cells harvested for experiments was assessed by Trypan Blue exclusion, and only populations of cells with viability >95% were used for experiments.

FIG. 1. Structures of glucocorticoid antedrugs (GCAs).

GC preparation

Dry powders of GCs No. 1 to 13, including 3 control compounds PRED, FPRED, and 21-acetyloxy-FPRED (Table 1), were dissolved and sonicated in ethanol and diluted in an RPMI-1640 medium to nanomolar (nM) concentration units for each cell treatment.

A549 alveolar type II epithelial cell drug treatment

Trypsin-dispersed HAE A549 cells were washed re-suspended in antibiotic free medium supplemented with 2% fetal calf serum, seeded at 2.5×10^5 cells/mL in 12-well cluster plates (1 mL/well), incubated, and allowed to attach overnight. In a series of preliminary studies, it was determined that maximal GCA effects were observed at 6 to 12 h. After treatment with different GCAs at different concentrations (300, 150, and 75 nM for each GCA), monolayer cells were washed twice with Hank's balanced salt solution, resuspended in RPMI-1640/F-12K for 6 h, and treated with or without different proinflammatory stimulants such as Th-2 type cytokine (IL-4; 100 ng/mL) for CCL26/eotaxin-3 protein expression and release, or Th-1-type cytokines (IL-1 β , tumor necrosis factor [TNF]- α , and interferon [IFN]- γ ; 50 ng/mL each) for CCL11/Eotaxin-1, or synergetic cytomix Th-1/Th-2-type cytokines (IL-4, IL-1 β , TNF- α , and IFN- γ ; 50 ng/mL each) for IL-8/CXCL8 and RANTES/CCL5 protein expression, and released in a serum-free medium for 24 h. After cytokine treatment, the cell culture supernatant was collected.

15 HL-60 eosinophilic cell drug treatment protocol

After EOS cell differentiation, the cells were plated 5×10^6 /mL in 12-well cluster plates (1 mL/well) and treated for 24 h with different GCAs at a low concentration, 75 nM. The small size of EOS cells and their characteristic as unattached cells in the plates make it difficult to extract a supernatant when treated with high concentrations of GCAs >75 nM. The treated cells were centrifuged (80 g, 10 min), washed twice in Hank's balanced salt solution, and resuspended in RPMI-1640 for 6 h. After being centrifuged and washed again, the cells were treated with or without Th-2-type cytokines (IL-4; 100 ng/mL) for Eotaxin-3/CCL26 or Th-1-Type cytokines cytomix (IL-1 β and TNF- α ; 50 ng/mL each) for IL11/Eotaxin-1, IL8/CXCL8, and RANTES/CCL5 in a fresh serum-free medium for 24 h. The cell culture supernatant was collected.

Quantification of cell proliferation, percentage of cell viability, and apoptosis of cells

After the cell culture supernatant was collected, the HAE and EOS cells were harvested in 1 mL of PBS containing Trypsin/EDTA. Trypan Blue was used to determine the percentage of cell viability, and proliferation was determined by cell counting with a hemocytometer. Apoptosis was assessed by visualization of EOS and HAE cell shrinkage, chromatin condensation, and cell fragmentation by microscopy.

Measurement of inflammatory markers

Synthesized and secreted CCL26, CCL11, IL-8, and RANTES proteins were detected using specific ELISA kits (R&D Systems). These ELISAs recognize both natural and recombinant human CCL26, CCL11, IL-8, and RANTES.

These chemokines were quantified with the Power Wa-veX340-I microplate reader equipped with KC4 v3.0 PowerReports software.

Data handling and analysis

All experiments were conducted in duplicate or triplicate as indicated in the figure legends and repeated on at least 3 to 4 separate occasions. Unless otherwise stated, all data are expressed as means $\pm$ standard error of the mean. When indicated, one-way analysis of variance followed by either the Tukey multiple comparisons or Dunnett post-test, as appropriate, was applied to experimental results to determine statistical significance (* P < 0.05; ** P < 0.01) between the indicated groups. The average of percent release of chemokine was determined by the following equation: [concentration of chemokines released after treatment with different drugs]/[concentration of chemokines released

FIG. 2. (A; Treatment -1, -3, -5, -7, -9, -12, Untreated cells and Cells + IL-4), (B; Treatment -2, -4, -6, -8, -10, -11, -13, Untreated cells and Cells + IL-4) Treatment of A549 alveolar type II airway epithelial cells with GCAs significantly decreases interleukin (IL)-4 stimulated CCL26/Eotaxin-3 protein release. Cells were treated for 6 to 12 h with different GCAs, and each compound was diluted to 3 different concentrations: 300, 150, and 75 nM. Treated cell monolayers were washed with a serum-free medium for 6 h and then incubated for an additional 24 h in the presence of Th-2-type cytokine IL-4 at 100 ng/mL. CCL26 was quantified in culture supernatants by specific sandwich ELISA. Data are mean $\pm$ standard error of the mean (SEM); $n \geq 3$. * P < 0.05 and ** P < 0.01 indicate significant difference between stimulated cells with IL-4 as the positive control and GC-treated cells. ♦ P < 0.05 and ♦♦ P < 0.01 indicate significant difference of protein release between cells treated with a same GCA derivative at 3 concentrations: 300, 150, and 75 nM. The average of percent release of chemokine was determined by the following equation: [concentration of chemokines released after treatment with different drugs]/[concentration of chemokines released after stimulation with cytokines only] $\times$ 100.

after stimulation with cytokines only]×100 (Fig. 2). The percent of inhibition was determined by subtracting the average percent of chemokine release from 100 percent, which represents the concentration of chemokines released after stimulation with cytokines only (Table 2). The average of percent of cell viability was determined by the following equation: [number of viable cells]/[total number of cells]×100. The average of percent of cell proliferation was determined by the following equation: [number of viable cells after treatment with different drugs]/[number of viable cells without treatment and after stimulated cells with cytokines only]×100.

Results

Effect of GCA treatment on stimulated HAE cell inflammation

Anti-inflammatory GCAs inhibit cytokine-induced eotaxin-3 (CCL26). Cultured HAE A549 cells were left untreated or treated with different concentrations of GCAs for 12 h. The cells then were washed free of the drug for 6 h and treated with or without different proinflammatory IL-4/Th-2-type cytokines for 24 h. HAE cells not treated with GCA constitutively released 209±15.3 pg/mL of CCL26. Stimulation of

TABLE 2. PERCENT INHIBITION OF STIMULATED CHEMOKINES (CCL26, CCL11, IL-8, AND RANTES) WITH TH-1- OR TH-2-TYPE CYTOKINES

No.	Compounds	[nM] of GCAs treatment	% of inhibition of chemokines in HAE cells with GCAs				No.	[nM] of GCAs EOS treatment	% of inhibition of chemokines in EOS cells with GCAs			
			CCL26	CCL11	IL-8	RANTES			CCL26	CCL11	IL-8	RANTES
1	P-ISO-21AC	300	84	54	15	59	1	75	72	83	74	92
		150	61	52	12	65						
		75	50	40	11	67						
2	FP-ISO-21AC	300	88	95	42	74	2	75	43	59	0	2
		150	78	71	43	79						
		75	61	84	40	79						
3	P-ISO-ET-21OH	300	35	91	19	22	3	75	64	69	78	93
		150	41	68	13	46						
		75	40	90	18	32						
4	FP-ISO-ET-21OH	300	71	85	32	46	4	75	62	43	2	6
		150	80	92	27	55						
		75	78	51	34	52						
5	P-ISO-ET-21AC	300	79	34	12	9	5	75	68	64	83	89
		150	79	60	4	12						
		75	47	33	7	13						
6	FP-ISO-ET-21AC	300	27	94	26	4	6	75	43	19	6	4
		150	74	87	24	32						
		75	69	36	28	56						
7	P-OXIM-21AC	300	88	93	31	45	7	75	48	55	0	7
		150	87	98	35	59						
		75	73	100	17	69						
8	FP-OXIM-21AC	300	85	97	22	55	8	75	64	43	1	15
		150	81	94	24	71						
		75	63	49	32	72						
9	P-OXIM-21OH	300	84	67	24	51	9	75	61	48	1	15
		150	91	66	13	63						
		75	79	56	7	43						
10	FP-OXIM-21OH	300	85	94	20	16	10	75	71	21	0	6
		150	35	90	21	10						
		75	38	48	21	47						
11	FP-21AC	300	77	82	39	41	11	75	58	63	1	8
		150	70	73	37	41						
		75	76	81	38	70						
12	PRED	300	85	55	27	51	12	75	27	28	1	11
		150	89	72	35	73						
		75	86	39	28	52						
13	FPRED	300	41	77	43	51	13	75	29	53	0	3
		150	57	92	41	71						
		75	55	87	46	79						

The percent of inhibition was determined by subtracting the average percent of chemokine release from one hundred per cent related to the positive control, stimulated cells only.

HAE, human airway epithelium; EOS, eosinophil.

these HAE cells with IL-4/Th-2-type cytokines increased the secretion of CCL26 to 968.9 ± 61 pg/mL (Fig. 2A, B).

The constitutive secretion level, therefore, was $\sim 22\%$ of the stimulated secretion level. All 3 concentrations (75, 150, and 300 nM) of PRED (compound No. 12) and FP-21AC (No. 11) inhibited IL-4-stimulated A549 cell CCL26 secretion to constitutive levels or below. FPRED (No. 13) was not as effective, inhibiting secretion only to 40%–60% of the stimulated secretion range.

Several of the GCAs inhibited IL-4-stimulated A549 cell CCL26 secretion as well as the PRED and FP-21AC, and for some, the inhibition was concentration dependent. The highest concentration (300 nM) of all the PRED-based compounds (No. 1, No. 5, No. 7, and No. 9) except No. 3 (P-ISO-ET-21OH) was as effective as PRED in inhibiting CCL26 secretion to constitutive levels or below. All 3 concentrations of No. 3 decreased secretion only to 60%–65% of the stimulated level. The lower concentration levels of No. 1 (75 and 150 nM) and No. 5 (75 nM) were also significantly less effective, decreasing secretion to only 50%–60% (Fig. 2A). Similar results were found with the FPRED-based compounds. The highest concentrations of all (No. 2, No. 4, No. 8, No. 10, and No. 11), except No. 6 (FP-ISO-ET-21AC), inhibited CCL26 secretion to constitutive levels or below. For No. 10, secretion inhibition by the lower concentrations (75 and 150 nM) was significantly less than the highest concentration. In addition, No. 6 was unusual in that the lower concentrations (75 and 150 nM) were significantly more inhibitory than the 300 nM concentration (Fig. 2B).

Anti-inflammatory GCAs inhibit cytokine-induced eotaxin-1 (CCL11). When HAE cells were stimulated with cytomix (IL- β , TNF- α , and IFN- γ) at 50 ng/mL, release of the chemokine CCL11 was 216 ± 11 pg/mL. Unstimulated HAE A549 cells released CCL11 at a constitutive level of 9.7 ± 3.9 pg/mL, which is $\sim 5\%$ of the stimulated secretion level (Fig. 3A, B).

All 3 concentrations of FPRED (No. 13) inhibited most of the stimulated secretion to 8%–23%. PRED (No. 12) was less effective overall and most inhibitory at 150 nM, which decreased CCL11 secretion to 28%. Of the PRED-based GCAs, P-OXIM-21AC (No. 7) was the most inhibitory; all 3 concentrations inhibited secretion to unstimulated levels or below. The next most inhibitory was No. 3, followed by No. 9 and No. 1. P-ISO-ET-21AC (No. 5) was the least inhibitory (Fig. 3A). For the FPRED-based GCAs, No. 2, No. 6, No. 8, and No. 10 at 300 nM inhibited CCL11 secretion to constitutive levels or below, and 150 nM of No. 4, No. 6, No. 8, and No. 10 was also highly inhibitory (Fig. 3B). The isoxazoline derivatives No. 2, No. 3, No. 4, and No. 6 and the oxime derivatives No. 7, No. 8, and No. 10 are all excellent inhibitors of CCL11 secretion.

Anti-inflammatory GCAs inhibit cytokine-induced IL-8 (CXCL8). HAE cells treated with cytomix (IL-4, IL- β , TNF- α , and IFN- γ) at 50 ng/mL increased the release of IL-8 to $1,210.38 \pm 18.7$ pg/mL in the supernatant compared to untreated cells in which the constitutive release was 129.51 ± 8.2 pg/mL, which is $\sim 11\%$ of the stimulated level (Fig. 4A, B). Both PRED (maximal inhibition to 65% of stimulated secretion) and FPRED (maximal inhibition to 54% of stimulated secretion) significantly inhibited stimulated IL-8 secretion, but neither inhibited secretion to the constitutive level. In general, the FPRED-based GCAs were slightly better inhibitors than the PRED-based GCAs (Fig. 4A, B).

FIG. 3. (A; Treatment -1, -3, -5, -7, -9, -12, Untreated cells and Cells +Cytomix), (B; Treatment -2, -4, -6, -8, -10, -11, -13, Untreated cells and Cells +Cytomix) Treatment of A549 alveolar type II airway epithelial cells with GCAs significantly decreases cytomix- (IL- β , TNF- α , and IFN- γ) stimulated CCL11/Eotaxin-1 protein release. Cells were treated for 6 to 12 h with different GCAs, and each compound was diluted to 3 different concentrations: 300, 150, and 75 nM. Treated cell monolayers were washed with a serum-free medium for 6 h and then incubated for an additional 24 h in the presence of Th-1-type cytokine cytomix (IL- β , TNF- α , and IFN- γ) at 50 ng/mL. CCL11 was quantified in culture supernatants by specific sandwich ELISA. Data are mean $\pm$ SEM; $n \geq 3$. * $P < 0.05$ and ** $P < 0.01$ indicate significant difference between stimulated cells + cytomix as the positive control and GC-treated cells. ♦ $P < 0.05$ and ♦♦ $P < 0.01$ indicate significant difference of protein release between cells treated with a same GCA derivative at 3 concentrations: 300, 150, and 75 nM. The average of percent release of chemokine was determined by the following equation: [concentration of chemokines released after treatment with different drugs]/[concentration of chemokines released after stimulation with cytokines only] $\times 100$.

Anti-inflammatory GCAs inhibit cytokine-induced RANTES (CCL5). The HAE cells treated with cytomix (IL-4, IL- β , TNF- α , and IFN- γ) at 50 ng/mL also increased the release of RANTES to 340 ± 10.2 pg/mL compared to untreated cells in which the constitutive release was 16.23 ± 5.3 pg/mL ($\sim 5\%$ of the stimulated release; Fig. 5A, B). FPRED at 75 nM (No. 13; 21% of stimulated secretion) and PRED at 150 nM (No. 12; 27% of stimulated secretion) inhibited secretion significantly better than the other concentrations of those compounds and better than most of the GCAs. Of the GCAs, the PRED-based No. 1, No. 7, and No. 9 were more inhibitory than No. 3 and No. 5, and the FPRED-based No. 2, No. 8, and No. 11 were somewhat more inhibitory than No. 4, No. 6, and No. 10. Intriguingly, for several of the GCAs in both groups [No. 6,

FIG. 4. (A; Treatment -1, -3, -5, -7, -9, -12, Untreated cells and Cells + Cytomix), (B; Treatment -2, -4, -6, -8, -10, -11, -13, Untreated cells and Cells + Cytomix) Treatment of A549 alveolar type II epithelial cells with GCAs significantly decreases cytomix- (IL-4, IL- β , TNF- α , and IFN- γ) stimulated IL-8/CXCL8 protein release. Cells were treated for 6 to 12 h with different GCAs, and each compound was diluted to 3 different concentrations: 300, 150 and 75 nM. Treated cell monolayers were washed with a serum-free medium for 6 h and then incubated for an additional 24 h in the presence of Th-1/Th-2-type cytokine cytomix (IL-4, IL- β , TNF- α , and IFN- γ) at 50 ng/mL. IL-8 was quantified in culture supernatants by specific sandwich ELISA. Data are mean $\pm$ SEM; $n \geq 3$. * $P < 0.05$ and ** $P < 0.01$ indicate significant difference between stimulated cells + cytomix as the positive control and GC-treated cells. The average of percent release of chemokine was determined by the following equation: [concentration of chemokines released after treatment with different drugs]/[concentration of chemokines released after stimulation with cytokines only] $\times 100$.

No. 7, No. 10, and No. 13 (FPRED)], the lowest concentration (75 nM) was more inhibitory than higher concentrations (Fig. 5A, B).

In general, the oxime-derivative GCA compounds No. 7 (69% of inhibition) and No. 8 (72% of inhibition) were significant inhibitors of RANTES release, and the isoxazoline derivative the GCA compounds No. 1 (59%–67% of inhibition) and No. 2 (74%–79% of inhibition) were also good inhibitors at different concentrations of RANTES secretion (Table 2).

Effect of GCA treatment on stimulated Clone 15 HL-60 EOS cell inflammation

Anti-inflammatory GCAs inhibit cytokine-induced eotaxin-3 (CCL26). EOS cells stimulated with the Th-2-type cytokine IL-4 released 599.8 ± 48.5 pg/mL of CCL26, more than a 3-fold increase over the constitutive level of secretion 171.34 ± 15 pg/

FIG. 5. (A; Treatment -1, -3, -5, -7, -9, -12, Untreated cells and Cells + Cytomix), (B; Treatment -2, -4, -6, -8, -10, -11, -13, Untreated cells and Cells + Cytomix) Treatment of A549 alveolar type II airway epithelial cells with GCAs significantly decreases cytomix- (IL-4, IL- β , TNF- α , and IFN- γ) stimulated RANTES/CCL5 protein release. Cells were treated for 6 to 12 h with different GCAs, and each compound was diluted to 3 different concentrations: 300, 150, and 75 nM. Treated cell monolayers were washed with a serum-free medium for 6 h and then incubated for an additional 24 h in the presence of Th-1/Th-2-type cytokine cytomix (IL-4, IL- β , TNF- α , and IFN- γ) at 50 ng/mL. RANTES was quantified in culture supernatants by specific sandwich ELISA. Data are mean $\pm$ SEM; $n \geq 3$. * $P < 0.05$ and ** $P < 0.01$ indicate significant difference between stimulated cells + Cytomix as the positive control and GC-treated cells. ♦ $P < 0.05$ and ♦♦ $P < 0.01$ indicate significant difference of protein release between cells treated with a same GCA derivative at 3 concentrations: 300, 150, and 75 nM. The average of percent release of chemokine was determined by the following equation: [concentration of chemokines released after treatment with different drugs]/[concentration of chemokines released after stimulation with cytokines only] $\times 100$.

mL ($\sim 29\%$ of the stimulated secretion; Fig. 6). Although neither 75 nM PRED nor FPRED significantly decreased IL-4-stimulated CCL26 secretion, most of the GCAs, No. 1, No. 3, No. 4, No. 5, No. 8, No. 9, No. 10, and No. 11, at 75 nM, inhibited secretion to the constitutive level (** $P < 0.01$), except No. 2 and No. 6 (* $P < 0.05$) (Fig. 6 and Table 2).

Anti-inflammatory GCAs inhibit cytokine-induced eotaxin-1 (CCL11). When stimulated with cytomix (IL-1 β and TNF- α), the EOS cells released 29.68 ± 3 pg/mL of CCL11 compared to 21.37 ± 3 pg/mL constitutively released from untreated cells ($\sim 72\%$ of stimulated release). Although the cytomix augmentation of CCL11 release was not significant (Fig. 7) and PRED and FPRED had no significant effect on CCL11 release, treatment with the isoxazoline GCA compounds No. 1, No. 2, No. 3, and No. 5 (59%–83% of inhibition) significantly inhibited CCL11 release. FP-21AC (No. 11) also significantly inhibited CCL11 release (63% of inhibition) (Table 2).

FIG. 6. Treatment of 15 HL-60 eosinophilic cells with GCAs significantly decreases IL-4-stimulated CCL26/Eotaxin-3 protein expression and release. Cells were treated for 24 h with different GCAs at concentration $[C]=75$ nM. Treated eosinophil (EOS) cells were washed with a serum-free medium for 6 h and then incubated for an additional 24 h in the presence of Th-2-type cytokine IL-4 at 100 ng/mL each. CCL26 was quantified in culture supernatants by specific sandwich ELISA. Data are mean $\pm$ SEM; $n \geq 3$. $*P < 0.05$ and $**P < 0.01$ indicate significant difference between stimulated cells with IL-4 as the positive control and cells stimulated and treated with GCAs. The average of percent release of chemokine was determined by the following equation: $[\text{concentration of chemokines released after treatment with different drugs}] / [\text{concentration of chemokines released after stimulation with cytokines only}] \times 100$.

FIG. 7. Treatment of 15 HL-60 eosinophilic cells with GCAs significantly decreases cytomix- (IL- β and TNF- α) stimulated CCL11/Eotaxin-1 protein expression and release. Cells were treated for 24 h with different GCAs at concentration $[C]=75$ nM. Treated EOS cells were washed with a serum-free medium for 6 h and then incubated for an additional 24 h in the presence of Th-1-type cytokine cytomix (IL- β and TNF- α) at 50 ng/mL each. CCL11 was quantified in culture supernatants by specific sandwich ELISA. Data are mean $\pm$ SEM; $n \geq 3$. $*P < 0.05$ and $**P < 0.01$ indicate significant difference between stimulated cells with cytomix (IL- β and TNF- α) as the positive control and cells stimulated and treated with GCAs. The average of percent release of chemokine was determined by the following equation: $[\text{concentration of chemokines released after treatment with different drugs}] / [\text{concentration of chemokines released after stimulation with cytokines only}] \times 100$.

FIG. 8. Treatment of 15 HL-60 eosinophilic cells with GCAs significantly decreases cytomix- (IL- β and TNF- α) stimulated IL-8/CXCL8 protein expression and release. Cells were treated for 24 h with different GCAs at concentration $[C]=75$ nM. Treated EOS cells were washed with a serum-free medium for 6 h and then incubated for an additional 24 h in the presence of Th-1-type cytokine cytomix (IL- β and TNF- α) at 50 ng/mL each. IL-8 was quantified in culture supernatants by specific sandwich ELISA. Data are mean $\pm$ SEM; $n \geq 3$. $**P < 0.01$ indicates significant difference between stimulated cells with cytomix (IL- β and TNF- α) as the positive control and cells stimulated and treated with GCAs. The average of percent release of chemokine was determined by the following equation: $[\text{concentration of chemokines released after treatment with different drugs}] / [\text{concentration of chemokines released after stimulation with cytokines only}] \times 100$.

Anti-inflammatory GCAs inhibit cytokine-induced IL-8 (CXCL8). Untreated EOS cells also constitutively released very high concentrations of IL-8 (754.3 ± 23 pg/mL), but not significantly different compared to 793.13 ± 2 pg/mL from cells stimulated with cytomix (IL-1 β and TNF- α) (Fig. 8). Only the isoxazoline derivative GCA compounds No. 1 (74% of inhibition), No. 3 (78% of inhibition), and No. 5 (83% of inhibition) significantly inhibited IL-8 release (Table 2). No inhibition activity was observed with any oxime derivatives, fluoroisoxazoline compounds, nor the GCA's molecular parents, No. 11 (FPRED-21AC), No. 12 (PRED), and No. 13 (FPRED) (Fig. 8 and Table 2).

Anti-inflammatory GCAs inhibit cytokine-induced RANTES (CCL5)

Untreated EOS cells constitutively released high concentrations of RANTES ($1,474.81 \pm 39$ pg/mL), but not significantly different compared to $1,485.26 \pm 32$ pg/mL from cells stimulated with cytomix (IL-1 β and TNF- α) (Fig. 9). Only the isoxazoline-derivative GCA compounds No. 1 (92% of inhibition), No. 3 (93% of inhibition), and No. 5 (89% of inhibition) significantly inhibited the release of RANTES (Table 2). There was no inhibition activity with oxime derivatives or fluoroisoxazoline derivatives, including the GCA's molecular parents No. 11 (FPRED-21AC), No. 12 (PRED), or No. 13 (FPRED) (Fig. 9 and Table 2).

HAE and EOS cell proliferation, viability, and apoptosis during GC treatment. There was a significant increase ($P < 0.01$) of 28% in cell proliferation for EOS cells stimulated with Th-1-

FIG. 9. Treatment of 15 HL-60 eosinophilic cells with GCAs significantly decreases cytomix- (IL- β and TNF- α) stimulated RANTES/CCL5 protein expression and release. Cells were treated for 24 h with different GCAs at concentration [C]=75 nM. Treated EOS cells were washed with a serum-free medium for 6 h and then incubated for an additional 24 h in the presence of Th-1-type cytokine cytomix (IL- β and TNF- α) at 50 ng/mL. RANTES was quantified in culture supernatants by specific sandwich ELISA. Data are mean $\pm$ SEM; $n \geq 3$. ** $P < 0.01$ indicates significant difference between stimulated cells with cytomix (IL- β and TNF- α) as the positive control and cells stimulated and treated with GCAs. The average of percent release of chemokine was determined by the following equation: [concentration of chemokines released after treatment with different drugs]/[concentration of chemokines released after stimulation with cytokines only] $\times 100$.

type cytokine cytomix (IL- β and TNF- α) compared to the untreated cells (Fig. 10). We observed 3 different groups of GCA activity on EOS cell proliferation: (1) GCAs No. 4, No. 6, No. 7, and No. 8 had no significant effect on cytomix-stimulated cells, (2) GCAs No. 2 (26%), No. 9 (24%), No. 10 (26%), No. 11 (46%), and No. 13 (43%) significantly reduced EOS cell number to levels well below the untreated cell control, and (3) GCAs No. 1 (76%), No. 3 (68%), No. 5 (68%), and No. 12 (61%) caused an even greater reduction to the EOS number compared to both untreated cells ($^{oo}P < 0.01$) and the Th-1-type cytokine cytomix-stimulated cells ($P < 0.01$).

The percent of EOS cell viability decreased significantly ($P < 0.01$) only after treatment with the nonfluorinated isoxazoline derivative GCAs, No. 1 (42.2%), No. 3 (53.8%), No. 5 (54.3%), and No. 12 (71.5%), and these treated cells displayed morphological evidence of injury after exposure. The percent of cell viability was more than 92% for EOS treated with GCAs No. 2, 4, 6, 7, 8, 9, 10, 11, and 13, untreated cells and cells stimulated with Th-1-type cytokine cytomix (IL- β and TNF- α), according to the Trypan Blue test. By microscopy, the morphology of the EOS cells treated with 75 nM of GCA compounds No. 1, No. 3, and No. 5 shows evidence of apoptosis, which included EOS cell shrinking, chromatin condensation, and cell fragmentation into discrete apoptotic bodies (Fig. 11, EOS cell treatment 1, 3, and 5). This apoptosis could account for the significant loss of EOS cells found with these treatments.

In HAE cells, only treatment with GCA No. 1 at concentrations of 300 and 150 nM significantly reduced ($P < 0.01$) the percentage of cell viability to 61% and 80%, respectively, reduced cell proliferation up to 51.4%, and caused apoptosis (Fig. 12, treatment 1). The percent of cell viability was more than 91% for HAE cells treated with GCAs No. 2, 3, 4, 5, 6, 7,

FIG. 10. GCA-induced effects of apoptosis and reduction of EOS cell proliferation. The EOS cell proliferation and quantification were determined after each GC cell treatment [75 nM] and stimulation with Th-1-type cytokine Cytomix (IL- β , TNF- α). Data are mean $\pm$ SEM; $n \geq 3$. ** $P < 0.01$ indicates significant difference between stimulated cells with cytomix (IL- β and TNF- α) as the positive control and cells stimulated and treated with GCAs. $^{oo}P < 0.01$ indicates significant difference between untreated cells as the negative control and cells stimulated and treated with GCAs.

8, 9, 10, 11, and 13, and cells stimulated with Th-1/Th-2-type cytokine cytomix (IL-4, IL- β , TNF- α , and IFN- γ).

Discussion

Th-1/Th-2 type cytokine stimulation

Cell inflammation is characterized by increased RNA expression and protein secretion of inflammatory cascades. These inflammatory proteins include cytokines, chemokines, growth factors, enzymes, receptors, and adhesion molecules (Barnes and others 1998a). In this study, increased secretion of chemokines CCL26, CCL11, CCL5, and IL-8 has been observed in activated HAE cells. These chemokines play a central role in causing chemotaxis of inflammatory cells, including T-cells, monocytes, neutrophil, and EOS cells, in the airway submucosa (Yousefi and others 1995; Berkman and others 1996; Ying and others 1999; Errahali and others 2009). IL-4, a type Th-2 cytokine, induces airway epithelial cell lines to rapidly secrete CCL26 into the media. IL-4 is a particularly critical cytokine in allergic responses and is produced at elevated levels in inflammatory tissue (Zimmermann and others 2003). Alveolar type II cells respond to IL-4 by upregulation of CCR3 and concomitant release of CCL26 with similar kinetics (Errahali and others 2009). TNF- α and IL-1 β induce the production and secretion of eotaxin-1, IL-8, and RANTES, and secretion of these 3 chemokines is enhanced by the addition of IFN- γ ; and IL-4 in airway epithelium cells. TNF- α and IL-1 β can be detected at increased levels in the lung lavage fluid of patients with asthma, and *in situ* hybridization studies have implicated airway epithelial cells as well as alveolar macrophages in IL-1 β production (Kraft and others 1995). TNF- α , IL-1 β and IFN- γ , Th-1-type cytokines, and IL-4 Th-2-type cytokine may cause the characteristic symptoms of asthma inflammation, despite this fact that they are not a potent chemoattractant to EOS. Lilly and others

FIG. 11. Effect of [75 nM] of GCA treatment on structure morphology development of EOS cells. Images of cells were captured after 48 h of treatment by a Nikon Eclipse microscope (40 $\times$ objective) fitted with a Celestron camera. Photographs were visualized using Adobe Photoshop. The circled cells in treatment 1, 3, and 5 show the signs of apoptosis.

(1997) suggest that effects of these cytokines on EOS recruitment are mediated by proinflammatory chemokines such as CCL26, CCL11, CCL5, and IL-8. These results imply that TNF- α or IL-1 β can synergize with IFN- γ produced by Th-1 cells to stimulate airway epithelium. TNF- α , IL-1 β , and IFN- γ can interact with cytokine IL-4 produced by Th-2 cells to induce HAE IL-8 and RANTES production and secretion. However, the secretion of an eotaxin is independent of the Th-2 lymphocyte products (Lilly and others 1997).

This study also describes the ability of human EOS to constitutively release high amounts of CCL11, RANTES, and IL-8, suggesting an active participation of these cells in an inflammatory response. Increased eotaxins, RANTES, and IL-8 released by inflammatory cells in the lungs of asthmatic patients may be responsible for the recruitment of additional inflammatory cells and might therefore enhance the inflammation symptoms that result in chronic inflammation and the occurrence of even more damaging cancer cells (Aggarwal and others 2006).

Concentration dependence and the potency of GCAs

GCs are anti-inflammatory steroids that can cause a decrement in airway eosinophilia, downregulation of airway chemokine and cytokine production and secretion, and res-

olution of asthma symptoms (Adelroth and others 1990; Burke and others 1992; Djukanović and others 1992; Laitinen and others 1992). The inhibition by the GC of cytokine-induced eotaxins (CCL11 and CCL26), RANTES, and IL-8 protein production in HAE cells constitutes an important mechanism by which cytokine-induced airway inflammation and chemokine upregulation are suppressed by the different structures of isoxazoline and oxime derivatives.

The variable inhibitory effect on the secretion of chemokines CCL26, CCL11, and RANTES are GCA concentration dependent. In HAE cells, the GCA compounds No. 1, 2, 4, 5, 7, 8, and 9 at high concentrations (300 and 150 nM) are potent inhibitors of CCL26 secretion from 79% to 91%; the GCA compounds No. 2, 3, 4, 6, 7, 8, and 10 at high concentrations are also potent inhibitors of CCL11 secretion from 91% to 97%; the GCA compounds No. 2, 4, 7, and 8 at all concentrations are significant inhibitors of IL-8 secretion from 32% to 42%; and the GCA compounds No. 1, 2, 7, and 8 at a low concentration (75 nM) are potent inhibitors of RANTES secretion from 59% to 79% (Table 2). The inhibition of eotaxins was affected mostly by high concentrations and RANTES by low concentrations of the GCA derivatives. More interestingly, the oxime compound No. 7 at a low concentration (75 nM) would be the best compromise for knocking down CCL26, CCL11, IL-8, and RANTES in HAE cells compared to its molecular parents. The oxime structure and acetyl group

FIG. 12. Effect of [300 nM] of GCA treatment on structure morphology development of alveolar airway epithelium cells A549. Images of cells were captured after 48 h of treatment by a Nikon Eclipse microscope (40 $\times$ objective) fitted with a Celestron camera. Photographs were visualized using Adobe Photoshop.

of compound No. 7 optimized the potency of the anti-inflammatory response of different chemokines and resulted in the best interaction with different GRs.

For the best outcomes, the dose of GCs should be cautiously monitored and adjusted to find the minimum effective dose. In human treatment, it is recommended to use the lowest dosage that will maintain an adequate clinical response. The degree to which the concentration, route, and duration of GC administration correlate with the specific risks related to infection and adverse systemic effects is still unclear. It has been reported that with high doses of GCs, the rate of occurrence of infectious complications increases (Minnecci and others 2004).

Many homeostatic mechanisms under the control of GRs can be both stimulated and inhibited. The main drawback of GCs is the potential for adverse side effects associated with systematic accumulation, due to the high concentration of doses, and nonspecific actions of GC treatment. Drug accumulations can cause important disruptions in the GC mechanisms and can lead to systemic side effects. For ex-

ample, the chronic administration of high doses of GCs can cause growth retardation or suppression of the hypothalamic-pituitary-adrenal axis (Lee and Soliman 1982; Lee and Ko 1999). In the current design of new effective and safe GCs, structural modification and manipulation are an important strategy for optimizing the potency and decreasing the concentration of GCs during the treatment of cell inflammation and asthma.

This study suggests that future experimentation should be done on small concentrations of a combination of 2 or 3 potent inhibitory compounds from isoxazoline and oxime derivatives. This combination could be a powerful drug inhibitor of cell inflammation. For example, different combinations of fluorinated and nonfluorinated isoxazoline and oxime compounds No. 1, 2, 7, and 8 may increase the percent reduction of many inflammatory chemokines, since they are the best compounds in CCL26, CCL11, IL-8, and RANTES inhibition in HAE cells. Additionally, the compounds No. 1, 2, 7, and 8 studied previously in the laboratory displayed higher anti-inflammatory activities than their molecular parents PRED and FPRED in an ear edema bioassay. High

affinity of most GCA compounds for GRs was observed, using the receptor-binding assay except No. 3 and No. 5, and the metabolism of the compounds No. 2 and No. 8 *in vitro*, which were found to be rapid, with half-lives of 2.1 and 4.2 min, respectively (Park and others 2006).

Eotaxin is thought to be important in recruiting EOS to the airway after allergic stimulation (Griffiths-Johnson and others 1993; Jose and others 1994; Rothenberg and others 1995b; Ganzalo and others 1996). Eotaxin is unique among EOS-active chemoattractants in that it specifically attracts EOS. This specificity has recently been demonstrated *in vitro* for both mouse (Rothenberg and others 1995a, 1996; Ganzalo and others 1996) and human (Garcia and others 1996; Ponath and others 1996b) during chemotaxis. Although eotaxin can be produced by several cell types, production in airway epithelial cells may be particularly relevant to allergic diseases, as eotaxin immunoreactivity is detectable in the epithelial cells of nasal polyps that are infiltrated with EOS (Ponath and others 1996a). In addition, RANTES, IL-8, and eotaxins have been shown to attract EOS to the lung in a coordinated manner at the development of airway inflammation in allergic mice (Ganzalo and others 1996). EOS cells have the capacity to produce, store, and release IL-8 similar to human neutrophils (Schröder and others 1989; Bazzoni and others 1991; McCain and others 1993; Takahashi and others 1993; Wertheim and others 1993; Wei and others 1994). The presence of EOS at the sites of allergic inflammation might release this constitutive intracellular IL-8 and RANTES (Rot and others 1992).

In the treated EOS cells with 75 nM of GCAs, we observed a wide range of potency in the inhibition of CCL26 and CCL11. More specifically, the nonfluorinated isoxazoline GCAs, No. 1, 3, and 5, were highly potent inhibitors to CCL26 (64%–72%), CCL11 (64%–83%), CCL5 (74%–83%), and IL-8 (89%–93%). However, these inhibitions were associated with EOS cell apoptosis (Fig. 11, EOS cell treatment 1, 3, and 5). This result confirms that GCA nonfluorinated isoxazoline derivatives induce the suppression of chemotaxis protein secretion, which causes the inhibitory effect on EOS migration to the site of inflammation, but also induces EOS cell apoptosis. Thus, apoptosis dramatically decreases the release of constitutive IL-8 and RANTES in airway EOS cells. Our previous experiments have demonstrated that inhibition of CCL26 chemokine secretion by specific small interfering RNA treatments reduces EOS migration and activation up to 80% and 90% (Errahali and others 2009).

HAE and EOS cell proliferation, viability, and apoptosis

Incomplete repair in cell inflammation may lead to the continued production of inflammatory cytokines and chemokines, which in turn may lead to further, undesirable effects in the airway cells (Aggarwal and others 2006). An increased number of blood and tissue EOS is frequently observed in patients suffering from bronchial asthma and atopic dermatitis, suggesting a role for the EOS in the pathogenesis of these diseases. Additionally, the EOS is capable of cooperative interactions and activation with other leukocyte cells (Del Pozo and others 1992; Hansel and others 1992; Weller and others 1993).

An important drug action of interest is focused on GC-induced inflammatory leukocyte cell apoptosis, including

the apoptosis of EOS cells. The GCA derivatives No. 2 and No. 11 significantly reduce the EOS cell proliferation compared to the stimulated cells only (Fig. 10). The GCA isoxazoline derivative No. 1 can cause severe EOS cell apoptosis at a low concentration (75 nM) in addition to the compounds No. 3 and No. 5 (Fig. 11, EOS cell treatment 1, 3, and 5). However, only high concentrations of more than 300 nM of GCA isoxazoline No. 1 can cause apoptosis, as well as shedding and change in the structural morphology of alveolar type II epithelium cells (Fig. 12, treatment 1). The confirmation *in vivo* is still required. Regardless of corticosteroid use, epithelial cells are seen in the bronchoalveolar lavage fluid of asthmatics of varying severity (Foresi and others 1990). Other studies show that corticosteroid-induced apoptosis in hematopoietic cells the in culture is rapidly taking place in <24 h (Jamieson and Yamamoto 2000). Still, the method of epithelial injury and repair is not clear. One possibility is that a high concentration and accumulation of potent corticosteroid therapy may contribute to cell toxicity and the shedding of resident cells, which permits side effects. In this study, the low concentration (75 nM) of isoxazoline compounds No. 1, 3, and 5 can cause apoptosis in EOS cells and significantly inhibit the proinflammatory chemokines CCL26, CCL11, IL-8, and RANTES in HAE and EOS cells during cell inflammation or asthma.

In summary, our data demonstrate 3 distinct therapeutic categories of GCAs: the first category of GCA contains various structures, including fluorinated isoxazolines (No. 4 and No. 6) and fluorinated and nonfluorinated oximes (No. 7 and No. 8), which significantly inhibit CC chemokine production and secretion of CCL26, CCL11, IL-8, and RANTES in HAE cells and only CCL26 in EOS cells. The second therapeutic class of GCA derivatives contains various structures, including fluorinated isoxazoline (No. 2), fluorinated PRED (No. 11 and No. 13), and nonfluorinated PRED (No. 12), which significantly reduce EOS cell proliferation and cause the inhibition of all CC chemokines, including CCL26, CCL11, IL-8, and RANTES in HAE cells, as well as CCL26 and CCL11 in EOS cells. The third class of GCAs are specifically nonfluorinated isoxazoline derivative structures (No. 1, No. 3, and No. 5), which cause EOS cell apoptosis and inhibit all CC chemokines in HAE and EOS cells.

Conclusion

Novel therapeutic groups of GCA derivatives can inhibit airway cell inflammation by several mechanisms, including alteration of CC chemokine and cytokine secretion in HAE and EOS, induction of EOS apoptosis, and limitation of EOS cell proliferation at the site of inflammation. Since these new GCAs do not exhibit the suppressive effect on the hypothalamus–adrenal axis at the effective anti-inflammatory doses, they could be useful agents as long-term antiasthmatics and at different severity levels of cell inflammation.

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Author Disclosure Statement

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