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The CCK_2 receptor antagonist, YF476, inhibits *Mastomys* ECL cell hyperplasia and gastric carcinoid tumor development

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ABSTRACT

YF476 is a potent and highly selective cholecystokin 2 (CCK_2) receptor antagonist of the benzodiazepine class. It inhibits gastric neuroendocrine enterochromaffin-like (ECL) cell secretion, proliferation and spontaneous formation of gastric neuroendocrine tumors (carcinoids) in cotton rats. The *Mastomys* rodent species exhibits a genetic predisposition to gastric ECL neuroendocrine tumor formation which can be accelerated by acid suppression and induction of hypergastrinemia. In this respect, it mimics the human condition of atrophic gastritis, hypergastrinemia and gastric carcinoid development. We investigated whether YF476 could inhibit acid suppression-induced ECL cell hyperplasia and neoplasia in this model. In addition, we examined whether YF476 could reverse established ECL cell hyperplasia and neoplasia. Targeting the CCK_2 receptor during Loxtidine-induced hypergastrinemia resulted in a reduction in ECL cell secretion (plasma and mucosal histamine, and histidine decarboxylase (*HDC*) transcripts, $p<0.05$) and proliferation (numbers of HDC-positive cells, connective tissue growth factor (*CTGF*) and *cyclin D1* transcription). This was associated with a decrease in ECL cell hyperplasia and a 60% reduction in gastric ECL cell microcarcinoid (tumors <0.3 mm in size) formation. YF476 inhibited ECL cell neoplasia (gastric carcinoid) in animals with hyperplasia, inhibited the formation of ECL cell tumors when co-administered with Loxtidine and reversed the growth and developement of gastric ECL cell carcinoids in long-term acid suppressed *Mastomys*. Variable importance analysis using a logistic multinomial regression model indicated the effects of YF476 were specific to the ECL cell and alterations in ECL cell function reflected inhibition of transcripts for *HDC*, *Chromogranin A* (*CgA*), CCK_2 and the autocrine growth factor, *CTGF*. We conclude that specifically targeting the CCK_2 receptor inhibits gastrin-mediated ECL cell secretion and ECL cell proliferation and tumor development *in vivo*.

1. Introduction

Gastrin, apart from its key regulatory role in gastric acid secretion, is a trophic hormone that causes gastric mucosal proliferation and has a putative influence on the development of gastrointestinal neoplasia [1,2]. Its predominant cellular targets in the oxyntic mucosa are cholecystokinin 2 (CCK_2) receptor expressing neuroendocrine enterochromaffin-like (ECL) cells. Gastrin, as well as modulating ECL cell histamine secretion, is also a potent trophic regulator of ECL cell proliferation.

Hypergastrinemia, as a consequence of acid blockade, culminates in ECL cell hyperplasia, and ultimately neoplasia (~2 years) in rodents [3,4]. This progresses gradually from diffuse, linear and micronodular hyperplasia to dysplasia, and if the hypergastrinemia persists, usually as a secondary response to drug-induced gastric acid inhibition [5–9], it may lead to the development of gastric neuroendocrine tumors (NETs) of ECL cell origin [3–5]. This effect is accelerated in the African rodent *Mastomys* (*Praomys natalensis*) [5,8,10] and in the spontaneously hypergastrinemic cotton rats (*Sigmodon hispidus*) [11,12]. The *Mastomys* develops benign neuroendocrine (ECL) cell tumors within two months of administration of irreversible H_2-receptor blockers or proton pump inhibitors [5,10]. Examination of this model has demonstrated that the growth effects of gastrin result in ECL cell proliferation and hyperplasia (0–10 weeks) [10]. By 12 weeks of sustained hypergastrinemia, ECL cell transformation becomes largely autonomous of gastrin [10] although some tumors may still exhibit gastrin-sensitive growth [13], while non-transformed ECL cells remain gastrin-sensitive and continue to develop into neoplasia. ECL cell proliferation is also responsive to stimulation by other growth factors [14] including connective tissue growth factor (CTGF) [15]. Gastrin stimulates ECL proliferation via MAPK signaling, AP-1 pathway activation and Cyclin D1 over-expression [16]. In addition,

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histamine produced by proliferating ECL cells, also plays a modulatory role in tumor growth [17–19].

Specific, efficient CCK_2 receptor antagonists including YF476 have been developed [20,21] which inhibit gastric acid secretion in rodents [20–22] and dogs [20,21] and may potentially be useful in the treatment of acid-related diseases [23]. *In vitro*, YF476 is a potent inhibitor of ECL cell function, specifically reducing histamine release ($IC_{50} = 1.2 \times 10^{-9}$ M) and proliferation ($IC_{50} = 1.3 \times 10^{-12}$ M) [24]. These effects are more potent (10–1000×) than other CCK_2 receptor antagonists, e.g. L365260, or targeting the ECL cell somatostatin receptors; the latter has been suggested as clinical treatment for hypergastrinemia-derived gastric NETs [25]. *In vivo*, YF476 induced gastric mucosal hypotrophy with reduced mucosal thickness under normogastrinemic conditions [26]. Under hypergastrinemic conditions, the combination of targeting CCK_2 and use of H_2 receptor antagonists, inhibited *Helicobacter*-induced gastric cancer in INS-GAS mice [27]. Similarly, a significant reduction in the occurrence (33% to 5.6%, $p = 0.04$) of spontaneous gastric (ECL cell-derived) carcinomas in 8 month old cotton rats has been demonstrated [28].

Based on these studies, we postulated that YF476 would inhibit gastrin-mediated ECL cell function (histamine secretion and proliferation). We therefore examined the hypothesis that YF476 would inhibit both gastrin-mediated ECL cell hyperplasia and neoplasia development in the *Mastomys* model of rapid gastric neuroendocrine neoplasia and secondly, whether YF476, could reverse established ECL cell hyperplasia and neoplasia. As a control, we also examined the effects of gastrin on EC cells, a cell that is known not to express CCK_2 receptors [29].

2. Methods

2.1. Test substances

The gastrin receptor antagonist YF476, (R)-1-[2,3-dihydro-2-oxo-1pivaloylmethyl-5-(2-pyridyl)-1H-1,4-benzo-diazepin-3-yl]-3-(3-methylaminophenyl) urea, was generously supplied by Trio Medicines Ltd, London, UK. It was resuspended in polyethylene glycol 300 at a final concentration of 0.25 mg/ml and a single subcutaneous injection (300 µmol/kg body weight) was administered to animals every month. At this concentration, YF476 blocks ECL cell CCK_2 receptors in rats for 8 weeks [22]. The dose and frequency of dosing has previously been effectively used in cotton rat studies [28]. Loxtidine (Glaxo, Ware) was administered using the standard treatment protocol, 1 mg/kg in drinking water [15,30].

2.2. Study design

A total of 19 animals (10 males, 9 females; median age 7 months: range 6–12 months) were treated with YF476 as indicated in the schema (Fig. 1). Three control groups (normal: $n = 14$; 8 week Loxtidine treated/hyperplasia: $n = 4$ and 16 week Loxtidine treated/tumor: $n = 4$) (14 males: 8 females; median age 7 months (range 6–12 months) were used to compare the different treatment protocols. To examine the question of whether YF476 prevented ECL cell neoplasia formation, we tested the effects of YF476 (16 weeks) on the development of ECL cell hyperplasia/neoplasia in animals treated with Loxtidine for 16 weeks ($n = 5$) (Group I). To examine the question of whether YF476 inhibited the transformation of ECL cells from hyperplasia, we examined the effects of this drug both in the presence of hypergastrinemia (Group II: 8 weeks of YF476 in the presence of ongoing (16 weeks total Loxtidine treatment: $n = 4$)) and in the absence of this drive (Group III: 8 weeks of YF476 in the absence of ongoing Loxtidine treatment (initial 8 weeks treatment only): $n = 5$)). Finally, we wanted to determine whether YF476 could reverse ECL cell neoplasia. To examine this, we determined the effects of YF476 (8 weeks) on animals with tumors (Group IV: 16 weeks Loxtidine treatment) ($n = 5$). At the termination of the study, animals were euthanized and gastric mucosa (formalin fixed-paraffin-embedded or frozen in TRIZOL) and plasma samples collected for analyses.

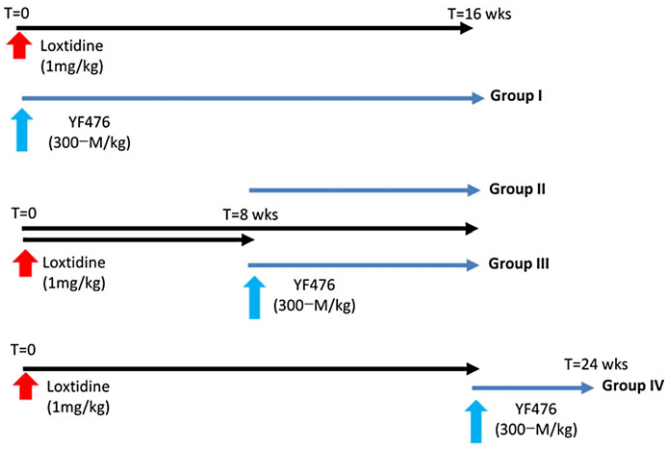

Fig. 1. Schema for Loxtidine-induced ECL cell proliferation and the four YF476 treatment groups. At time 0 ($T = 0$), Loxtidine (1 mg/kg) was given to animals in drinking water. In Group I, animals underwent 16 weeks of Loxtidine treatment. They also underwent 16 weeks of concomitant YF476 treatment (YF476 applied at $T = 0$). Group II underwent 8 weeks of Loxtidine, and at $T = 8$ weeks, YF476 was introduced. Group III underwent 8 weeks of Loxtidine treatment. This was then stopped and animals underwent 8 weeks treatment with YF476 (in the absence of Loxtidine). Finally, Group IV consisted of animals that underwent 16 weeks of Loxtidine treatment. This was then stopped and animals were treated with YF476 for an additional 8 weeks.

2.3. Histopathology

Serial (5 µm) sections from paraffin-embedded *Mastomys* gastric mucosa were stained with hematoxylin and eosin (H&E). Two observers (blinded to the original code — MK and RLC) jointly classified samples as normal, hyperplasia (linear or nodular hyperplasia) or tumor (dysplasia: cellular atypia/glandular distortion). Tumors <0.3 mm in size that were not macroscopically evident were described as microcarcinoids. If the mucosa displayed areas of both tumor and hyperplasia, it was classified as tumor. In each section, the number of cells in 10 gastric glands was counted. Mucosal counts were not undertaken in animals with microscopic gastric tumors.

For immunohistochemistry, sections were dual-stained with anti-histidine decarboxylase (HDC) and DAPI as previously described [17,19]. Briefly, after antigen retrieval (10 mM sodium citrate, pH 6.0), slides were incubated (24 h/4 °C) with 1:20 rabbit anti-HDC (MBL International). Goat anti-rabbit antibodies conjugated to a horseradish peroxidase-decorated dextran polymer backbone (RDI, Concord MA) were used as a secondary reagent to identify HDC immunoreactivity. This signal was visualized with a fluorescent chromogen (Cy5-tyramide; NEN Life Science Products, MA). Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI — 20 mg/ml). As a control, anti-HDC antibodies were excluded, and slides incubated with secondary antibodies and DAPI. HDC-positive cells were counted (using a magnification of 400×) and expressed at the percentage of total DAPI-positive cells. Five randomly selected points of properly orientated oxyntic mucosa were counted.

2.4. Gastrin and histamine ELISAs

Plasma and mucosal gastrin and histamine were measured using a commercially available gastrin ELISA (Assay Designs) or histamine ELISA assays (Rocky Mountain Diagnostics) according to manufacturer's instructions for serum samples [10,19,31]. For the mucosa, total protein was extracted by homogenization in lysis buffer and quantitated (Bio-Rad Protein Assay) as described [29]. Gastrin (pg/ml) or histamine (ng/ml) content of each sample was then measured and defined as pg gastrin or ng