Despite structural similarity, the five subtypes comprising the cholinergic muscarinic family of G protein-coupled receptors regulate remarkably diverse biological functions. This mini review focuses on the closely related and commonly co-expressed M1R and M3R muscarinic acetylcholine receptor subtypes encoded respectively by CHRM1 and CHRM3. Activated M1R and M3R signal via Gq and downstream initiate phospholipid turnover, changes in cell calcium levels, and activation of protein kinases that alter gene transcription and ultimately cell function. The unexpectedly divergent effects of M1R and M3R activation, despite similar receptor structure, distribution, and signaling, are puzzling. To explore this conundrum, we focus on the gastrointestinal (GI) tract and liver because abundant data identify opposing effects of M1R and M3R activation on the progression of gastric, pancreatic, and colon cancer, and liver injury and fibrosis. Whereas M3R activation promotes GI neoplasia, M1R activation appears protective. In contrast, in murine liver injury models, M3R activation promotes and M1R activation mitigates liver fibrosis. We analyze these findings critically, consider their therapeutic implications, and review the pharmacology and availability for research and therapeutics of M1R and M3R-selective agonists and antagonists. We conclude by considering gaps in knowledge and other factors that hinder the application of these drugs and the development of new agents to treat GI and liver diseases.

**Keywords:** muscarinic receptors, G protein-coupled receptors, gastrointestinal physiology, gastrointestinal disease, liver disease, cancer

**INTRODUCTION**

Muscarinic receptors (MRs) are class A (Rhodopsin-like) guanine nucleotide protein-coupled receptors (GPCRs) differentiated from other cholinergic receptors by preferential binding of muscarine rather than nicotine (Eglen, 2012; Tiwari et al., 2013). MRs are further subcategorized into five subtypes, designated M1R through M5R and encoded by CHRM1-CHRM5, each of which modulates a range of parasympathetic activities (Caulfield and Birdsell, 1998). These functionalities depend on tissue and membrane localization (Koenig and Edwardson,
Like other GPCRs, MRs are characterized by seven transmembrane helices designated TM1 through TM7, forming a partially-spiral configuration within the cell membrane (Hulme et al., 2003). Acetylcholine (ACh) binds on the extracellular aspect of MRs in a pocket formed by TM3, TM6, and TM7 residues. The five MR subtypes share 82–92% transmembrane region homology, with 64–82% sequence similarity overall (Maeda et al., 2019). As GPCRs, activated MRs interact with heterotrimeric guanine nucleotide-binding proteins (G-proteins), classified by their α subunits, to initiate downstream targets.

Although classically responsive to ACh, MRs, like other GPCRs, possess allosteric binding sites for naturally occurring and engineered non-ACh ligands, with varying degrees of preference; allosteric effects may result in surprising downstream actions in cell types not previously considered responsive to muscarinic signaling (Tolaymat et al., 2019). ACh and these “non-traditional” ligands provide MRs with the ability to modulate a broad repertoire of cells and biological systems including those associated with neuronal signaling, immune function, and cell trafficking, proliferation, and differentiation (Wessler and Kirkpatrick, 2008; McLean et al., 2016). Dysregulated post-MR signaling is associated with unregulated cell proliferation and cancer progression (Chen et al., 2019), an “overactive” bladder (Abrams et al., 2006), autoimmune diseases (Berg et al., 2010; Lee et al., 2013) and psychiatric disorders (Scarr, 2012; Vakalopoulos, 2014; Jeon et al., 2015). In addition to the discovery that non-traditional ligands can modify MR function, the production and release of ACh is more widespread than originally thought; a wide variety of non-neuronal cells express choline acetyltransferase (ChAT), the key enzyme needed to convert acetyl CoA and choline into ACh (Wessler and Kirkpatrick, 2012). Colon cancers, for example, express high levels of ChAT (Cheng et al., 2008). The variety of processes modulated by MRs has invited extensive research into the potential use of agonists, antagonists, and allosteric modulators for myriad disorders.

**Muscarinic Receptor Distribution and Post-Receptor Signaling**

MRs are expressed by a wide variety of tissues and cell types and control key digestive and metabolic functions. Salivary gland secretion, gastric, and intestinal fluid transport, cell proliferation, mucus production, motility, and mesenteric vascular constriction and dilation are all responsive to MR signaling (Tobin et al., 2009; Muise et al., 2017). In the stomach, M3R, M4R, and M5R activation modulates hydrochloric acid secretion from parietal cells (Aihara et al., 2005), and M1R and M2R activation stimulates pepsinogen secretion from chief cells (Xie et al., 2005). GI motility, through intestinal smooth muscle cell action, involves communication between the central and enteric nervous systems. These effects are partially mediated by M2R through M4R (Moro et al., 2005), with M2R and M4R playing a role in regulating longitudinal muscle contraction, and all three MR subtypes involved in circular muscle function (Harrington et al., 2010; Tanahashi et al., 2021). MR-mediated regulation of smooth muscle function extends throughout the entire GI tract. Nonetheless, it is likely that MRs play additional roles in regulating small intestinal function; for example, M2R is expressed in the stem cell compartment and may be involved in enterocyte turnover (Muise et al., 2017). ACh has both pro- (Koyama et al., 1992; Brunn et al., 1995) and anti-inflammatory (Pavlov and Tracey, 2006) effects, the latter mediated in part by reducing systemic levels of tumor necrosis factor. While the ubiquity of MRs within the digestive tract makes them attractive therapeutic targets to modulate health and disease, this same ubiquity complicates efforts to design selective agents while minimizing off-target adverse effects.

Responses of MRs to ligand binding are subtype specific. Activation of odd-numbered MRs (M1R, M3R, and M5R) stimulates phospholipid turnover and increases intracellular calcium levels while activation of even-numbered MRs (M2R, M4R) inhibits adenylyl cyclase activity, thereby reducing levels of intracellular cAMP. M1R, M3R, and M5R (MRodd) canonically couple to Gαq11 which induces the phospholipase C-mediated hydrolysis of phosphatidylglycerol into diacylglycerol and inositol (1,4,5)-trisphosphate. The latter binds an endoplasmic reticulum receptor stimulating intracellular calcium release. However, these may represent oversimplifications; experimental findings suggest differential interactions of individual MRodd and MEReven with their downstream targets. For example, although both M1R and M2R signal through phospholipase C, CHO cells expressing M1R exhibited four-fold greater cAMP production in response to carbachol compared to cells expressing M2R (Burford et al., 1995). Likewise, although M1R and M3R (MReven) act primarily by binding Gαq family proteins to alter adenylyl cyclase activity, their actions can also prolong potassium channel opening, thereby causing cellular hyperpolarization (Bubser et al., 2012).

These general principles do not tell the whole story—despite substantial sequence homology among MR subtypes they demonstrate surprising individuality in their responses to stimuli, even within the same cell and when responding to the same ligand. Pancreatic acinar cells provide a useful model to study muscarinic control of exocrine digestive function. Using acinar cells prepared from M1R- and M3R-deficient mice as well as M1/M3 chimeric receptors, Nakamura et al., demonstrated greater ACh-induced IP3 release in cells expressing only M1R compared to those expressing uniquely M3R (Nakamura et al., 2013). Moreover, in M1R-compared to M3R-expressing cells, these differences were associated respectively with oscillatory versus monotonic patterns of cytosolic calcium release. Oscillatory calcium release was a function of a C-terminal region of M3R with considerable variability among MR subtypes (Nakamura et al., 2013). In murine gastric chief cells, both M1R and M3R mediate pepsinogen secretion—deletion of either MR subtype reduces and combined M1R and M3R deficiency ablates cholinergic agonist-induced proenzymes secretion (Xie et al., 2005). Thus, in some cell types, MRodd have overlapping functions whereas in other cell types, MR subtype signaling appears divergent. In addition to the influence of their cell and tissue localization, other mechanistic differences between MR subtypes result in sometimes-opposing...
effects. GPCRs, including MRs, can also undergo “pre-coupling”, wherein a stable multimeric complex is present before ligand binding. Unlike other MR subtypes, M1R and M3R pre-couple with G\textsubscript{i/o} G-proteins, their non-preferential G protein, thereby potentially altering downstream effects (Jakubík et al., 2011).

Crystal structures of inactive M\textsubscript{1}R subtypes provide some insight into different allosteric and orthosteric binding sites (Kruse et al., 2012; Thal et al., 2016), but our understanding of the resulting functional differences between MR subtypes continues to evolve.

### Table 1: FDA/EMA approved muscarinic receptor antagonists and agonists.

| Generic (Trade) name | Activity/MR selectivity | Dose range/Route | Approved indications |
|----------------------|------------------------|-----------------|----------------------|
| Benztropine (Cogentin) Bolden et al. (1992) | M\textsubscript{1}R Ant | 0.5–6 mg/day IM/IV/PO | Parkinson’s disease, extrapyramidal symptoms, dystonia |
| Biperiden (Akineton) Eltze and Figala (1988) | M\textsubscript{1}R Ant | 1–16 mg/day PO, 2.5–5 mg IM/IV | Parkinson’s disease, extrapyramidal symptoms |
| Dicyclomine (Bentyl) Giachetti et al. (1986) | M\textsubscript{1}R Ant | 20–160 mg/day PO | Irritable bowel syndrome |
| Pirenzepine (Gastrozep) Bolden et al. (1992) | M\textsubscript{1}R Ant | 100–150 mg/day PO | Peptic ulcer disease |
| Trihexyphenidyl (Artane) Giachetti et al. (1986) | M\textsubscript{1}R Ant | 5–15 mg/day PO | Parkinson’s disease |
| Cevimeline/AF-102B (Evoxac) Weber and Keating (2008b) | M\textsubscript{1}, M\textsubscript{3}R Agonist | 90 mg/day PO | Xerostomia in Sjogren’s syndrome |
| Oxybutynin (Ditropan) Andersson and Chapple (2001) | M\textsubscript{1}, M\textsubscript{3}R Ant | 5–30 mg/day PO; topical and transdermal | Overactive bladder |
| Acclidinium (Tudorza Pressair) Beier et al. (2013) | M\textsubscript{1}R Ant | 800 mcg/daily inhaled | Chronic obstructive pulmonary disease |
| Darifenacin (Enablex) Yamada et al. (2006) | M\textsubscript{1}R Ant | 7.5–15 mg/day PO | Overactive bladder |
| Solifenacin (VESIcare) Oki et al. (2005) | M\textsubscript{1}R Ant | 5–10 mg/day PO | Overactive bladder |
| Aceclidine* (Glaunorm) Erickson and Schroeder (2000) | NS Agonist | Topical | Glaucome |
| Bethanechol (Urecholine) | NS Agonist | 30–200 mg/day PO | Urinary retention |
| Methacholine | NS Agonist | 1–380 mcg | Bronchial airway hyperactivity |
| Pilocarpine (Salagen, Isopto Carpine) Zimmerman (1981) | NS Agonist | 15–30 mg/day PO | Xerostomia, glaucoma |
| Atropine (Atropen) | NS Ant | 0.5–3 mg IV/IM; available as inhalant | Bradycardia, inhibit secretions; mushroom/organophosphate poisoning |
| Scopolamine (Transderm-Scop) | NS Ant | 1.5 mg skin patch; available PO, IM, IV | Nausea, sedation, GI and genitourinary spasm |
| Tolterodine (Detrol) Hills et al. (1998) | NS Ant | 2–4 mg/day PO | Overactive bladder |

**Note:** Ant, antagonist; EMA, European Medicines Agency; FDA, United States Food and Drug Administration; IM, intramuscular; IV, intravenous; NS, nonselective; PO, oral. *, not FDA approved.

### Table 2: Selective M\textsubscript{1}R/M\textsubscript{3}R agents used for research and under clinical investigation.

| Agent | Activity/MR selectivity | Source | Potential clinical applications |
|-------|------------------------|--------|-------------------------------|
| 2′ biaryl amides Budzik et al. (2010) | M\textsubscript{1}R Agonist | GlaxoSmithKline | Alzheimer’s disease, schizophrenia |
| 77-LH-28-1 Langmead et al. (2008) | M\textsubscript{1}R Agonist | | |
| AC-42 Henrich et al. (2009) | M\textsubscript{1}R Agonist | | |
| HTL0018318 Bakker et al. (2021) | M\textsubscript{1}R Agonist | Sosei Heptares Therapeutics | Dementia |
| PP5 Wodd et al. (2017) | M\textsubscript{1}R Agonist | AstraZeneca | Analgesia |
| Nitrocaramiphen Hudkins et al. (1993) | M\textsubscript{1}R Ant | | |
| PIPE-307 | M\textsubscript{1}R Ant | Pipeline Therapeutics | Multiple sclerosis; clinical trials (NCT04941781, NCT04725175) |
| PIPE-359 Schrader et al. (2021) | M\textsubscript{1}R Ant | Pipeline Therapeutics | |
| Telenzepine Evelleigh et al. (1989) | M\textsubscript{1}R Ant | Theracos | Peptic ulcer disease; obesity (Clinical trial NCT01155531) |
| VU 025503S Tsentsevitsky et al. (2017) | M\textsubscript{1}R Ant | Vanderbilt University | Seizure disorder |
| L-689,660 Hargreaves et al. (1992) | M\textsubscript{1}, M\textsubscript{3}R Agonist | | |
| Oxotremorine Veena et al. (2011) | M\textsubscript{1}, M\textsubscript{3}R Agonist | | |
| RZHEJU Hsu et al. (2012) | M\textsubscript{1}, M\textsubscript{3}R Ant | | Non-small cell lung cancer |
| McN-A-343 Mitchelson (2012b) | M\textsubscript{1}, M\textsubscript{3}R Agonist | | |
| Xanomeline Heinrich et al. (2009) | M\textsubscript{1}, M\textsubscript{3}R Agonist | | Alzheimer’s disease |
| 4-DAMP Honda et al. (2007) | M\textsubscript{1}R Ant | | |
| AZD8871 Aparici et al. (2019) | M\textsubscript{1}R Ant | Almirall | Chronic obstructive lung disease |
| DA-8010 Lee et al. (2019) | M\textsubscript{1}R Ant | | Overactive bladder |
| DAU 5884 Gosens et al. (2004) | M\textsubscript{1}R Ant | | |
| J-104129 Mitsuya et al. (1999) | M\textsubscript{1}R Ant | Merck | Obstructive airway disease |
| Temverine Kikuaka et al. (1998) | M\textsubscript{1}R Ant | | Urinary incontinence |
| YM905 Kobayashi et al. (2001) | M\textsubscript{1}R Ant | Astellas (Yamanouchi) | Irritable bowel syndrome |
| Arecoline Heinrich et al. (2009) | NS Agonist | | Alzheimer’s disease, schizophrenia |

**Note:** Ant, antagonist; NS, nonselective.
Effects of Dysregulated M1R and M3R Signaling on Non-Proliferative Disorders Involving the Digestive System

As a result of their central role in maintaining homeostasis in the GI tract, dysregulated MR signaling can be an important modifier of intestinal disease. In Hirschsprung disease, lack of mucosal cholinergic innervation in aganglionic colon segments increases the risk of postoperative enterocolitis (Keck et al., 2021). In diarrhea-predominant irritable bowel syndrome (IBS-D) without a concomitant psychiatric disorder, pyridostigmine (an acetylcholinesterase inhibitor) induces a stronger IL-6 response that is highly correlated with symptoms (Dinan et al., 2008). Given the pharmacotherapies targeting MRs already approved or being explored to treat IBS (Tables 1, 2), achieving a more precise mechanistic understanding of the role MR dysregulation plays in IBS is important.

Diseases associated with MR dysregulation are not restricted to the lower GI tract. In the stomach, cholinergic signaling is balanced with histamine and gastrin release to regulate gastric acid levels; peptic ulcer disease is associated with greater MR expression in the gastric body, whereas progressive MR loss in that region is associated with chronic gastritis (Pfeiffer et al., 1995). In progressive systemic sclerosis and Sjögren’s syndrome, an autoimmune condition which impairs lacrimal and salivary function, esophageal dysmotility may be associated with anti-M3R antibodies (Goldblatt et al., 2002; Kawaguchi et al., 2009; Gyger and Baron, 2012); anti-M3R antibodies are also reported in progressive systemic sclerosis with anal dysmotility (Singh et al., 2009; Gyger and Baron, 2012). Intravenous immunoglobulin to neutralize anti-M3R antibodies may be beneficial (Smith et al., 2005).

Compared to the normal liver, individuals with primary biliary cholangitis (PBC) are more likely to have a CHRM3 single nucleotide polymorphism (rs4620530) of uncertain significance; this is not associated with baseline disease characteristics or treatment responses (Greverath et al., 2020). PBC is more commonly associated with anti-M3R antibodies than other liver diseases (Tsuboi et al., 2014); those with anti-M3R antibodies are more likely to have a benign disease course. Nonetheless, M3R antibody levels do not correlate with treatment responses or serological markers either at baseline or during the disease course (Mayer et al., 2020). A subset of patients with PBC develop Sjögren’s syndrome; the shared increase in anti-M3R antibody levels in both conditions suggests overlapping features could form the basis for a mutual treatment.

Divergent Effects of M1R and M3R Signaling on Digestive Tract Cell Proliferation and Neoplasia

MRs play key roles in normal cell proliferation and turnover. As reviewed by Campoy et al. (2016), presumably to benefit tumor progression, neoplastic cells hijack MR-dependent proliferative signal transduction pathways. Treating neoplastic cells with exogenous ACh and inhibiting ACh hydrolysis promotes their proliferation and, conversely, reducing M3R expression and activation is anti-proliferative. Moreover, because neoplastic cells tend to lose cellular polarity, receptors normally expressed on the basolateral membrane may be expressed more diffusely around the cell membrane, thereby facilitating their access to orthosteric and allosteric ligands in the tumor microenvironment and GI lumen (Cheng et al., 2002). For example, bile acids, at concentrations achieved in stool, promote atrope-inhibitable colon cancer cell proliferation (Cheng and Raufman, 2005).

Abundant data support the conclusion that M1R plays an important role in colon cancer progression. In mouse models of sporadic and genetic colon cancer, using azoxymethane (AOM)-treated and Apc<sup>dmin</sup> mice, respectively, ChrM3 ablation with resulting M3R deficiency substantially reduces the intestinal tumor burden (Raufman et al., 2008; Raufman et al., 2011). As M3R deficiency primarily reduces the number of adenocarcinomas rather than adenomas, the major impact of blocking M3R activation appears to be on promotion, rather than initiation, of neoplasia. M3R activation has similar pro-proliferative effects on gastric cancer (Hayakawa et al., 2017; Wang et al., 2018). M1R expression is enhanced in cholangiocarcinoma and associated with reduced cell differentiation, perineural invasion, and metastasis (Feng et al., 2012; Feng et al., 2018).

In contrast to the impact of M3R deficiency, M1R deficiency in mice does not attenuate, and may modestly enhance, AOM-induced colon carcinogenesis. Strikingly, mice with combined M1R and M3R deficiency develop as many colon tumors as control mice (Cheng et al., 2014); that is, M3R deficiency negates the anti-neoplastic effects of M1R deficiency. Likewise, M1R agonism appears protective against pancreatic ductal adenocarcinoma (PDAC) and counteracts enhanced carcinogenesis following vagotomy (Renz et al., 2018), suggesting a potential therapeutic opportunity. In contrast, in hepatocellular and prostate carcinomas, M1R activation promotes cellular migration and invasiveness (Yin et al., 2018; Zhang et al., 2020). Notably, many of these studies are limited by using global rather than conditional knockout mouse models. Hence, it remains uncertain whether the respective MR deficiencies are due to effects on neoplastic cells versus other cellular elements in the tumor microenvironment (e.g., immunocytes). Nonetheless, these observations argue strongly for the importance of MR subtype selectivity in designing and developing therapeutics.

Branches of the vagus nerve, a major source of ACh signaling within the GI tract, innervate the liver and modulate hepatocyte regeneration by progenitor cells and fibrosis by stellate cells (Cassiman et al., 2002). The current lack of effective antifibrotic therapies highlights the potential of leveraging these muscarinic actions to prevent or reverse fibrosis in advanced liver disease and stimulate hepatocyte regeneration. For example, in rodents, carbon tetrachloride (CCL4)-induced hepatic fibrosis can be attenuated by vagotomy and treatment with atrope (Lam et al., 2008). M3R expression and activation protects against AOM-induced liver fibrosis (Khurana et al., 2010; Khurana et al., 2013; Rachakonda et al., 2015). Surprisingly, M3R expression and activation appears to have opposite effects,
worsening AOM-induced hepatic fibrosis (Rachakonda et al., 2015). Thus, in the absence of effective anti-fibrotic therapy, manipulation of MR subtype activity to limit or reverse fibrosis may have therapeutic potential although, again, divergent effects in different tissues warrants caution.

Use of MR Agonists and Antagonists to Treat Digestive Tract Disease

MR subtype, tissue distribution, and off-target side effects have hindered efforts to manipulate MR activity precisely and effectively with drugs. MR antagonists are most effective in treating chronic obstructive pulmonary disease and overactive bladder (Table 1) (Eglen et al., 1999; Athanasopoulos and Giannitas, 2011)—their utility for GI and hepatic disorders is currently limited. Cholinesterase inhibitors that increase ACh levels, also used clinically for digestive tract disorders, have similar limitations as their actions are largely non-selective. Adverse effects with these classes of drugs are attributed primarily to off-target effects on the CNS (e.g., convulsions, confusion) and other peripheral MR subtypes (e.g., salivary glands, rhinitis, diaphoresis, diarrhea, nausea, vomiting, and bronchospasm). Novel MR agonists and antagonists are currently under investigation primarily for diseases of the central nervous system such as Alzheimer’s disease and schizophrenia (Table 2) (Davie et al., 2013).

Several MR agonists and cholinesterase inhibitors are in clinical use. Oral and topical pilocarpine, and cevimeline (Evoxac), an M3R-selective agonist, augment salivary gland secretions in xerostomia due to radiation therapy and Sjögren’s syndrome (Iga et al., 1998; Fife et al., 2002; Petrone et al., 2002; Weber and Keating, 2008a; Berk, 2008; Mitchelson, 2012a; Davies and Thompson, 2015; Panarese and Moshibar, 2021). Bethanechol, a structural analogue of ACh that resists hydrolysis by cholinesterases, has potential to treat esophageal dysmotility. Currently approved to treat urinary retention and neurogenic bladder (Gaitonde et al., 2019), bethanechol strengthens esophageal contractions in subjects with ineffective esophageal motility (Agrawal et al., 2007) and augments lower esophageal sphincter pressure in gastroesophageal reflux disease (Farrell et al., 1973). Nonetheless, in a pilot study, topical bethanechol did not significantly improve esophageal motility (O’Rourke et al., 2013). Edrophonium, a cholinesterase inhibitor used to diagnose myasthenia gravis, was used to provoke esophageal spasm in the investigation of non-cardiac chest pain, but the lack of correlation between symptoms and objective changes in esophageal manometry limited its utility (Botoman, 2002).

Gastric acid secretion is controlled by a mix of cholinergic muscarinic stimulation and hormonal signaling by gastrin and histamine; thus, only partial inhibition of acid release is achieved with anti-muscarinic agents. Consequently, histamine-2 receptor and H+-K+ATPase (proton pump) inhibitors are highly successful and MR antagonists rarely prescribed. Pirenzepine (Gastrozepin), an M3R antagonist that is not FDA approved, has limited use to treat acid-related disorders in the EU (Tryba and Cook, 1997). Scopolamine, a non-selective MR antagonist, is commonly used as a transdermal patch for nausea associated with anesthesia or motion sickness (Riad and Hithe, 2021).

Dicyclomine (Bentyl), an M3R- and M4R-selective antagonist that inhibits small and large intestinal motility, is used as an anti-spasmodic agent to treat IBS (Giachetti et al., 1986; Doods et al., 1987). Neostigmine, a cholinesterase inhibitor, is used to treat acute intestinal pseudo-obstruction associated with critical illness or opioid use, another condition of impaired smooth muscle motility. Colonic decompression may be achieved with intravenous neostigmine (De Giorgio et al., 2001), although cardiac monitoring is important and rapid administration of atropine may be required for resulting bradycardia.

Although a potential role for modulating MR activity to treat cancer was demonstrated in a variety of cell types (Shah et al., 2009), except for an ongoing trial to investigate the utility of bethanechol before surgery for resectable PDAC (U.S. National Library of Medicine, 2021), the efficacy of modulators of MR activity in digestive tract cancers has not been tested in the clinic. Moreover, anti-tumor efficacy may be limited by the inability to achieve adequate concentrations in target tissues while, at the same time, preventing off-target adverse effects. An ideal agent would exhibit target organ and MR-subtype specificity, goals hampered by the extensive similarity between orthosteric and allosteric ligand binding sites among the five MR subtypes (Liu et al., 2018). Studies of naturally occurring ligands, such as muscarinic toxins in snake venom, have provided insight into how subtype-selective agents may be formulated (Maeda et al., 2020). Such agents with potential for oncotherapy continue to be developed. For example, the M4R-specific antagonist darifenacin which is approved to treat bladder dysfunction (Yamada et al., 2006) reportedly inhibits tumor progression and invasiveness in human-derived cell lines, most recently in colorectal cancer cell lines (Her et al., 2021). As darifenacin is in clinical use with a known safety profile, it is an attractive candidate for adjunctive therapy, especially for cancers already shown to overexpress M3R, like colon cancer cells (Frucht et al., 1999; Cheng et al., 2014), PDAC (Zhang et al., 2016), and non-small cell lung cancer (Lin et al., 2014).

Some therapeutic approaches may circumvent the need for MR subtype and tissue specificity. For example, treating colorectal cancers with poorly absorbed oral agents or drugs with extensive first-pass metabolism may target GI mucosal lesions with limited systemic side effects. However, even within a limited area of distribution, MRs are not constrained to only one downstream signaling pathway; the same receptor may have contradictory effects on neighboring cell types. Even when occupying the same binding pocket, ligands can influence the activation of pathways on other cell membrane surfaces via signaling bias and functional selectivity (Randákóva and Jakubík, 2021). A ligand may bind several MR subtypes, but only activate one or a few, thereby compensating for binding pocket homogeneity. Furthermore, through selective interactions with residues in the binding pocket of a single subtype, ligands can encourage activated receptor configurations that favor interaction with certain G proteins. As an example, the MR agonist cevimeline increased intracellular calcium levels in Chinese hamster ovary (CHO) cells transfected with rat M3R but did...
not increase cAMP levels. In contrast, carbachol, a non-selective MR agonist, elevated both calcium and cAMP levels (Gurwitz et al., 1994). Even more intriguing, cevimeline did not activate signaling in M3R-transfected cells, contrary to its clinical use in Sjogren’s syndrome which is thought to be mediated by M3R activation. This complexity makes it difficult to predict the clinical effects of new MR agonists and antagonists but suggests highly selective agents can be developed.

**CONCLUSION: CURRENT GAPS IN KNOWLEDGE, DRUG DEVELOPMENT, AND THERAPEUTIC OPPORTUNITIES**

MR activation via the vagus nerve, the longest and most complex cranial nerve, and within the enteric nervous system, is a major modifer of normal and pathological GI and hepatic function. As reviewed here, MRs and the machinery needed to produce their ligands are not limited to neuronal cells. Abundant evidence exists that “non-traditional” ligands (e.g., other than ACh) mediate paracrine and autocrine signaling by orthosteric and allosteric interactions with MR subtypes. These findings highlight the potential for treating a broad range of physiological and disease processes with MR subtype-selective agents. Numerous non-selective and subtype-selective orthosteric ligands that modify MR signaling have been developed and investigated to treat a variety of digestive diseases (Tables 1, 2); allosteric regulation of MR activity represents a presently untapped reservoir of agents that can be designed or repurposed to alter cell function. Overall, there has been limited clinical use of both orthosteric and allosteric modifiers of MR function. Despite more than 20 years of evidence supporting an important role for MR activation in GI cancer progression, currently only one clinical trial is investigating the efficacy of a drug to modulate MR activity as adjunctive treatment for a digestive tract cancer, PDAC (ClinicalTrials.gov Identifier: NCT03572283).

Extensive sequence homology between the five MR subtypes hampers efforts to create agents with sufficiently selective actions and, thereby, limited off-target toxicity. Adding to this complexity is the observation that a receptor subtype on one cell type may activate different downstream signaling pathways, depending on the interaction between ligand and receptor and the conformational changes instigated by this interaction. In addition to subtype-specificity, ideal agents must possess sufficient tissue specificity to prevent deleterious action on neighboring and distant tissues. In this regard, targeting diseases involving intestinal mucosa, e.g., neoplasia, may be advantaged by developing agents with limited GL absorption or extensive first-pass metabolism. Current gaps in knowledge include a better understanding of subtype-selective allosteric modulation of MR function, an area in its infancy.

Lastly, several observations reviewed above suggest great potential for leveraging the divergent actions of M1R and M3R activation to treat GI cancers. Thus, a drug design challenge is to develop a molecule with dual functionality as an M1R agonist and M3R antagonist. Moreover, it has not escaped our attention that developing an agent with the opposite properties may be useful to prevent or reverse hepatic fibrosis. Success at creating dual agonists for different bile acid receptors in the gut suggests that although the challenge is formidable, it can be overcome (Ito et al., 2021). As our understanding of these complex signaling mechanisms evolves and the medicinal chemistry needed to develop MR subtype-specific agents progresses, targeting MR subtypes is likely to become a valuable adjunct for treating a variety of digestive tract disorders, including cancer.

**AUTHOR CONTRIBUTIONS**

MT and JR conceptualized and wrote the initial draft. MS, MA, and GX proofread, edited, and contributed additional material. MT and JR completed the final draft.

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