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ORIGINAL ARTICLE

In pursuit of P2X3 antagonists: novel therapeutics for chronic pain and afferent sensitization

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Abstract Treating pain by inhibiting ATP activation of P2X3-containing receptors heralds an exciting new approach to pain management, and Afferent's program marks the vanguard in a new class of drugs poised to explore this approach to meet the significant unmet needs in pain management. P2X3 receptor subunits are expressed predominately and selectively in so-called C- and Aδ-fiber primary afferent neurons in most tissues and organ systems, including skin, joints, and hollow organs, suggesting a high degree of specificity to the pain sensing system in the human body. P2X3 antagonists block the activation of these fibers by ATP and stand to offer an alternative approach to the management of pain and discomfort. In addition, P2X3 is expressed presynaptically at central terminals of C-fiber afferent neurons, where ATP further sensitizes transmission of painful signals. As a result of the selectivity of the expression of P2X3, there is a lower likelihood of adverse effects in the brain, gastrointestinal, or cardiovascular tissues, effects which remain limiting factors for many existing pain therapeutics. In the periphery, ATP (the factor that triggers P2X3 receptor activation) can be released from various cells as a result of tissue inflammation, injury or stress, as well as visceral organ distension, and stimulate these local nociceptors. The P2X3 receptor rationale has aroused a formidable level of investigation producing many reports that clarify the potential role of ATP as a pain mediator, in chronic sensitized states in particular, and has piqued the interest of pharmaceutical companies. P2X receptor-mediated afferent activation has been implicated in inflammatory, visceral, and neuropathic pain states, as well as in airways hyperreactivity, migraine, itch, and cancer pain. It is well appreciated that oftentimes new mechanisms translate poorly from models into clinical efficacy and effectiveness; however, the breadth of activity seen from P2X3 inhibition in models offers a realistic chance that this novel mechanism to inhibit afferent nerve sensitization may find its place in the sun and bring some merciful relief to the torment of persistent discomfort and pain. The development philosophy at Afferent is to conduct proof of concept patient studies and best identify target patient groups that may benefit from this new intervention.

Keywords P2X3 receptor · P2X2/3 receptor · P2X3 antagonist · Anti-hyperalgesic · Analgesic · Joint pain · Visceral pain · Neuropathic pain

Abbreviations

ATP	Adenosine-5'-triphosphate
BBB	Blood–brain barrier
BPS	Bladder pain syndrome
CC	Chronic cough
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
CNS	Central nervous system
DAP	Diaminopyrimidine
DRG	Dorsal root ganglion
HTS	High throughput screening
IC	Interstitial cystitis
LUTS	Lower urinary tract symptoms
mIOA	Monoiodoacetate
Mrgpr	Mas-related G-protein coupled receptor
NCE	New chemical entity
OA	Osteoarthritis
OAB	Overactive bladder

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PNS	Peripheral nervous system
POC	Proof of concept
PPADS	Pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate
SAA	Substituted arylamide
SG	Stellate ganglion
SNI	Spared nerve injury
SNL	Spinal nerve ligation
TNP-ATP	2',3'-*O*-(2,4,6-trinitrophenyl)-ATP

Introduction

In pharmaceutical terms, we continue to fall short in addressing the increasing need for novel, effective, safe, and well-tolerated treatments for chronic pain and related conditions, despite decades of innovation and effort. Several classes of pain medicines are currently available, with each class displaying varied effectiveness for treating the multiple types of pain conditions. The four major classes are opioids, NSAIDs (including COX-2 inhibitors), anticonvulsants, and antidepressants. Additional minor classes (such as triptans, α_2-agonists, and local anaesthetics) have more limited and specific uses for certain (mostly acute) pain or related conditions. The limitations to clinical effectiveness for the major classes are extremely well known, and range from abuse liability, respiratory depression, and constipation (opioids), to gastrointestinal and cardiovascular harm (NSAIDs), to a myriad of negative central nervous system (CNS) effects (anticonvulsants and antidepressants). Added to these are the fundamental issues of limited efficacy (all classes) or tolerance development (opioids), and convenience issues such as need for dose titration (opioids, antidepressants, and anticonvulsants) and large, frequent doses (anticonvulsants). In all these cases, the concerns grow over chronic use and associated harm, and the potential for abuse that together widen the chasm between demand and available response.

There have been many efforts launched in pharmaceutical discovery for breakthrough of new analgesics and antihyperalgesics during the last three decades, but so far, none of these has delivered on the significant demands for registration, and thus the unmet need remains poorly resolved. These efforts have covered a selection of exciting novel chemicals directed at a range of diverse targets, such as 5-hydroxytryptamine 3 antagonists, tachykinin antagonists (e.g., NK1), calcitonin gene-related peptide (CGRP) antagonists, transient receptor potential vanilloid-1 antagonists, cannabinoid agonists, inter alia, but with little real clinical success, so far. Other relatively innovative approaches are still being pursued (e.g., channel-specific and state-dependent inhibitors of sensory pathway Na^+ or Ca^{2+} channels) but have been notable for substantial chemical difficulties.

A persistent challenge relates to the type of intervention sought for management of chronic pain. In contrast to acute, rapid action analgesia, where the goal is to suppress the perception of "nociceptive" pain arising from frank tissue injury (e.g., from acute infections, contusions, fractures, or surgery), in chronic pain situations, it is the pain itself that has often become largely pathological. In that sense, one has to attempt to target specifically the mechanisms leading to a change from *normal* to *abnormal* sensory perception, i.e., the mechanisms contributing to wind-up and sensitization that are associated with chronic and inappropriate sensations of pain to normally non-noxious or mildly noxious stimuli (allodynia and hyperalgesia, respectively). By the same token, the clear mandate is also to leave normal "defensive" pain perception intact and aim to suppress only those elements that specifically contribute to sensitization in tissues and organs, assuming they exist separately. Any treatment that reduces hyperalgesia but also leaves the individual partially insensitive to harmful mechanical or thermal insults will quickly erode its usefulness. By extension therefore, approaches to pain target discovery based on genetic sensory defects that produce, for example, congenital insensibility to pain, would seem a little off the ideal path.

P2 purinoceptor targeting

Medicines' discovery has evolved significantly over the last 20 years, with innovation and clinical differentiation more important than ever, and "me-too" drugs, formulation enhancements, and nth generations in class seem to be facing increasing challenges gaining approval and reimbursement. We have failed to reverse the continued decline in drug approvals—especially for new chemical entities (NCEs) and new mechanism agents for alleviation of many troubling chronic symptoms including discomfort and pain. The promise of genomics did not materialize it seems: genomic and genetic data, so easy as they are to come by, are so often examined not as part of a "totality" of information, but outside of the context of prevailing clinical physiology, pharmacology, and pathobiology—evolving knowledge garnered over the decades. Within this challenging context, the therapeutic targeting of purinergic signaling represents a rich and underdeveloped area. The purinergic field did not simply materialize overnight on the back of technological advances or fashions: it emerged in a gradual manner, intelligent and controversial, with occasional bursts of discovery. It is founded upon an abundance of diverse data unfolding over the last 40 years, with many classical pharmacological underpinnings, fostered by leading scientists in many countries (see [1]).

ATP is, of course, found abundantly in all tissues and cells, and seems to be overrepresented in pathological

milieu, with extensive literature militating towards the functional relevance of ATP in many disease processes. We now know that a multitude of cell surface receptors mediates the signaling functions of nucleotides in essentially every tissue and cell type, and again their contribution to function seems to be strongly regulated within pathological situations. These are the components upon which therapeutic advances have been often founded—multidisciplinary, form and function, pertinent to disease, translatable to clinical science. As such, continued progress in developing differentiated medicines at P2Y12 purinoceptors, the first examples of P2 receptor therapeutics, reflect these components with the successive advancement of therapeutically important antiplatelet agents: clopidogrel, ticlopidine, cangrelor, and ticagrelor. Other P2 receptor targets will likely be harnessed also for therapeutic benefit in the coming years.

The targeted inhibition of ATP-gated cation channels, the P2X receptors, has received significant focus from academic and pharmaceutical scientists in the quest of small molecule medicinal candidates, and the status of advancement—as can be gleaned from the literature and patents—was nicely reviewed in the recent past [2]. The P2X7 homotrimeric channel has clearly seen most medicinal progress, with many pharmaceutical companies successfully identifying developable small molecule antagonists to target a variety of inflammatory processes where ATP activation of immune-derived cells via P2X7 is purported to contribute to disease and symptom progression [3–5]. Several novel molecules are reported to have entered clinical studies (Pfizer's CE-224535; Astra-Zeneca's AZD-9056), with a small number of studies completed in patients with inflammation (e.g., rheumatoid arthritis (RA), chronic obstructive pulmonary disease (COPD)). Reports of modest efficacy have been observed with P2X7 antagonism in RA patients, which at the very least confirms the persistent presence of elevated ATP within inflamed joints at levels that must be fairly high, given the low sensitivity of the P2X7 receptor to ATP, as compared with other P2X ion-channels [6]; however, subsequent investigations in RA have apparently failed to show significant differentiation [7]. Nevertheless, additional interest in this target remains with among other things an exciting rationale for treatment of neuropathic pain conditions [8].

Beyond P2X7, there has been *significant* progress within the P2X family only in chemical efforts to target specifically ATP-gated channels that contain the P2X3 subunit, the P2X3 homotrimeric (P2X3.P2X3.P2X3), and P2X2/3 heterotrimeric (P2X2.P2X3.P2X3) receptors. This review now focuses on the rationale within a range of important sensory conditions, as well as the progress made in advancing novel medicinal candidates as "P2X3 antagonists" (i.e., blocking all P2X3 containing-channels), which have advanced to the level where patient studies are underway.

ATP and P2X3 relevance to primary afferent signaling and sensitization: indication rationales

A sensory role for ATP can be traced back over 50 years [9] to studies showing that ATP released from sensory nerves during antidromic stimulation caused vasodilation in the rabbit ear artery. It was considered possible that multiple purinergic pathways and receptors may be involved in the sensory actions of ATP; however, since their discovery in 1995 [10, 11], a crucial role has been proposed for receptors containing P2X3 subunits (homotrimeric P2X3 and heterotrimeric P2X2/3 receptors) in mediating the primary sensory effects of ATP [12–14]. P2X3 and P2X2/3 receptors are predominantly localized on small-to-medium diameter C- and Aδ-fiber sensory neurons within the dorsal root ganglion (DRG) and cranial sensory ganglia [15–17], and on their peripheral nerve terminals in receptive fields in tissues including the skin, joints, and viscera (Fig. 1). P2X3 and P2X2/3 receptors are also present on the central projections of these primary sensory neurons within the dorsal horn of the spinal cord and in the brainstem, where they apparently play a role in augmenting release of glutamate (as well as substance P) at this first sensory synapse [18–22]. Beyond these cellular locations, expression of P2X3 subunits appears to be somewhat sparse, with reports implicating certain epithelial cell populations (e.g., within the urinary bladder) and brainstem neurons as hosts, though with limited functional corollary described so far [23, 24].

Data supporting a therapeutic potential of selective antagonism of P2X3 containing receptors covers many investigational approaches: distribution of receptor subunit RNA and proteins; results from gene-targeting methods in mice and rats and studies of novel antagonists in preclinical rodent models; observations from human investigations of ATP content of pathological fluids and samples; expression of P2X3 immunoreactivity and studies of the effects of ATP itself in healthy volunteers or in isolated tissue samples. The range of potential therapeutic opportunities covers essentially any condition wherein chronic dysregulation and sensitization of populations of sensory neurons has been implicated, including inflammatory, visceral, neuropathic and cancer pain, as well as many conditions where bothersome "irritative" symptoms surface from similar chronic sensitization of afferent pathways (e.g., overactive bladder, irritable bowel syndrome, chronic itch and cough, and airways hyperreactivity). The evidence category that now remains is the key one: *what effects are observed in clinical studies using chemical antagonists for P2X3 containing receptors*? Clinical findings to date from studies

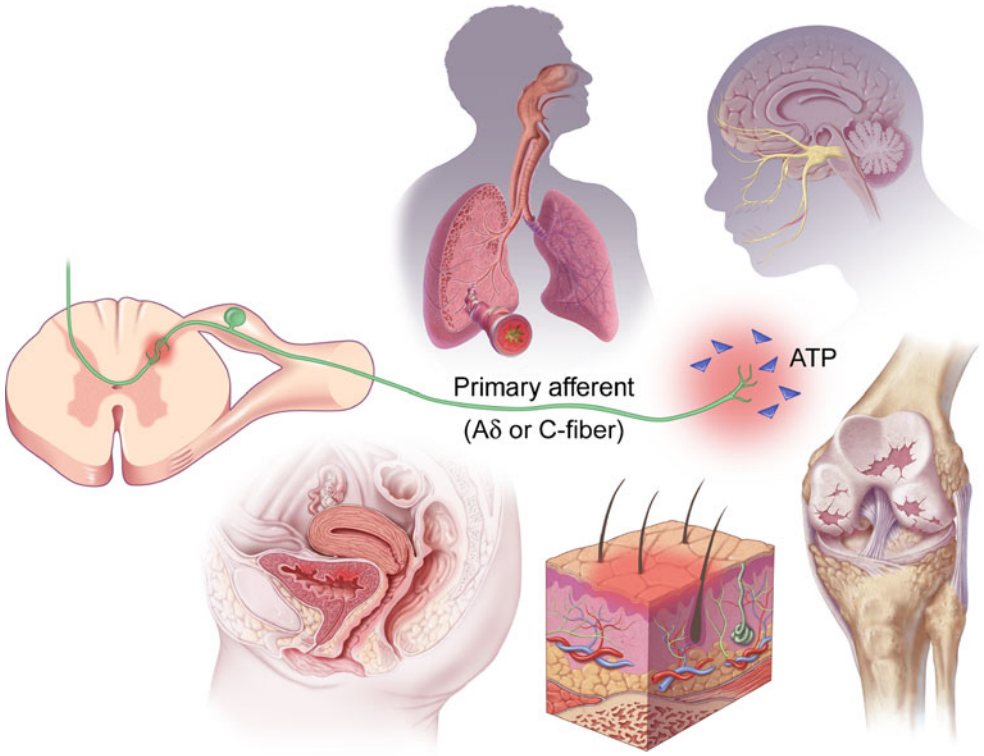

Fig. 1 P2X3 containing ionotropic receptors are found in a large proportion of unmyelinated and thinly myelinated primary afferent nerves innervating essentially all tissues and organs. ATP is released from many cell types in these receptive fields, as well as at the central terminals of activated afferents, and more so under conditions of injury, inflammation, stress, movement, and distension. Afferent sensitization results and appears to contribute to the serious symptoms of acute and chronic pain and irritation in musculoskeletal, visceral and neuropathic sensory disorders

with AF-219 (the only antagonist known to advance to human studies) have not been published, but in due course this dearth of information will change significantly.

A selection of reports over the last 40 years indicated the direct involvement of ATP in the perception of pain, including the early clinical demonstration that ATP applied to a blister base in healthy human volunteers was associated with heightened pain sensation [25–27]. ATP applied to forearm skin by iontophoresis also caused mild painful responses that were enhanced by sensitization with UV irradiation or intradermal capsaicin [28]. Intracutaneous injection of ATP [29], or its direct infusion into skeletal muscle [30], also caused significant pain in human volunteers. Subsequent studies in animals using more selective pharmacological and gene-based tools fortify the link between these effects and a crucial role for P2X3 and P2X2/3 receptors. Using the P2X1, P2X3, and P2X2/3 selective antagonist 2',3'-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) [31–35], and the P2X3, P2X2/3 selective antagonist A-317491 [36, 37], many investigators have shown that peripheral and spinal P2X3 and P2X2/3 receptors are involved in persistent, chronic neuropathic and inflammatory pain. Mice deficient in P2X3, P2X2, or both receptor subunits [38–40], as well as animals treated with P2X3-selective antisense [34, 41, 42] or short interfering RNA (siRNA) [43] revealed comparable findings. Collectively, these data have elucidated P2X3 as the principal substrate connecting ATP with chronic sensitization, especially in the context of injury and inflammation, yet, notably, with no clear evidence for involvement in the acute (defensive) sensation of noxious stimuli. Thus, a proposition that antagonism of P2X3 and/or P2X2/3 receptors may have potential therapeutic utility in the management of chronic pain conditions is reasonable. The following sections provide an overview of several indication areas where data provide strong clues to potential utility.

Inflammatory, joint, and musculoskeletal pain

ATP has a broad range of activities in inflammatory pathways, and through many different cell types and receptors that have been implicated in chronic inflammatory diseases [44–46]. However, evidence for inflammatory cell P2X3 receptor expression or activation by ATP is not readily apparent, and while sensory axon reflexes following activation of primary afferent neurons may give rise to release of factors (e.g., prostanoids, substance P or CGRP) that could contribute to or modulate neurogenic inflammatory responses, there is little evidence available specifically. Two reports have described possible functions of P2X3 receptors in activation of inflammatory and trophic responses in chondrocytes and synovial fibroblasts in joints [47, 48] but with no in vivo correlate, and thus there is little clear evidence that underlying joint disease progression is under the influence

of P2X3. In the context of symptom modification however, what happens to sensory responsiveness in tissues in models of chronic irritation or inflammation has been well studied, and there is clearly potential for utility of P2X3 antagonism in inflammatory pain conditions, including those in the musculoskeletal systems [36, 49–51].

Increased P2X3 receptor expression and function have been reported in several rodent joint models typified by application of noxious irritant substances or mechanical damage to paw, knee, vertebral, or temporo-mandibular sites [49–52]. Complete Freund's adjuvant (CFA), formalin, carrageenan, and mono-iodoacetate (mIOA), when injected acutely each gives rise to development of pain-related (nocifensive) behavior in rodents, such as reduced tolerance thresholds to mechanical and thermal provocation, reduced weight-bearing activity, as well as complex behaviors more relevant to emotional and cognitive responses to pain perception [53]. In these models, P2X3 receptor mRNA or expression are elevated in small- and medium-sized neurons in associated dorsal root or cranial ganglia, as well as in both the peripheral terminal fields in the joint and/or central terminals in dorsal horn of spinal or brainstem projections. Knock-out (KO) mice with deletions of P2X3, P2X2 and both receptor subunits showed reduced response to formalin injection into the paw [38, 39], while rats treated intrathecally with both antisense oligonucleotides as well as siRNA probes showed considerable reductions in mechanical and thermal hyperalgesia in classical paw or knee joint irritation models [34, 41, 43].

ATP content may also be elevated in inflamed or damaged tissues and joints [54] (Fig. 2), is able to activate arthritic knee-joint afferent fibers [55] and is present in arthritic patient synovial fluid [56, 57] as well as at tumor sites [58]. Recently, it was reported that in patients with arthritic knee joints [59], synovial ATP content was both positively associated with symptom severity and seen to decline during symptom ameliorating therapy with intra-articular hyaluronic acid.

Pharmacological approaches have been broadly used in most types of inflammatory and joint irritation models, and shown effective reduction in mechanical and thermal hypersensitivity following use of prototypic (non-specific) antagonists

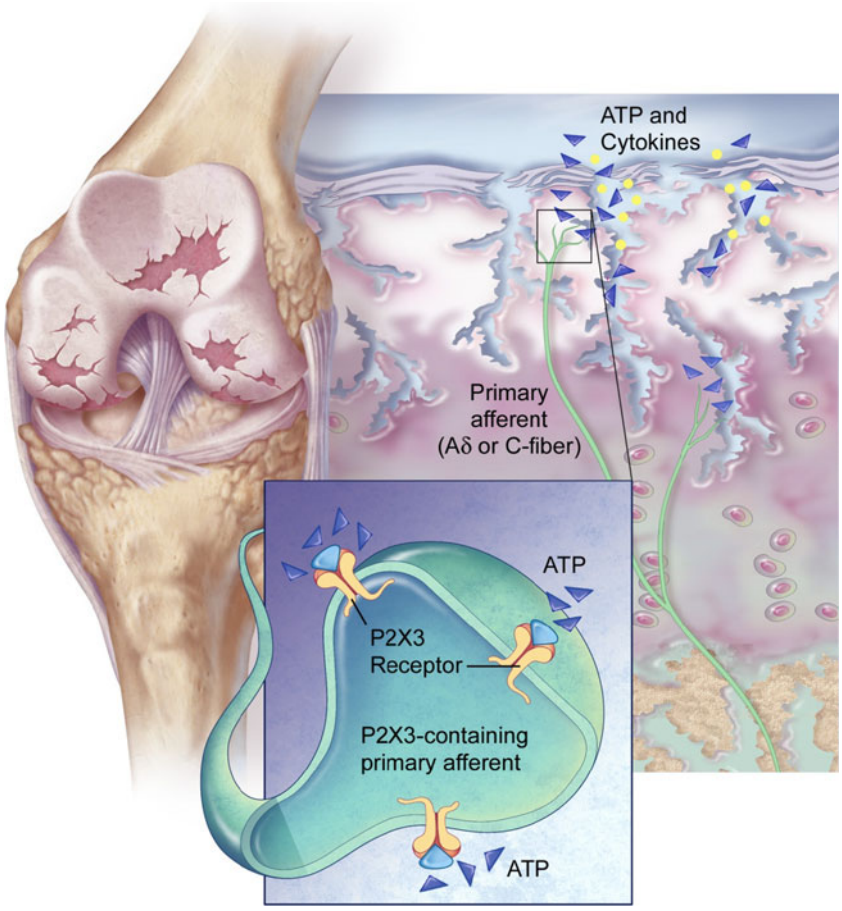

Fig. 2 Pain from damaged and strained joints and other musculoskeletal structures chronically impacts the lives of a vast number of individuals. Available pain medicines are often inadequately effective, poorly tolerated or considered unsafe, highlighting the great demand for medicines with novel mechanisms of action. P2X3 antagonists have shown to be effective in reversing hyperalgesia in several animal models of joint pain, and in studies of pain from cancer invasion of bony tissues. Osteoarthritis of the knee, an exceptionally common condition, is one of several high potential indications for novel P2X3 antagonists

(pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), suramin), selective polyanionic antagonists (TNP-ATP, A-317491) as well as novel selective drug-like antagonists. The most extensive examinations have utilized the tricarboxylic acid P2X3 antagonist A-317491, which due to its restricted permeability is useful for investigation following various parenteral routes of administration. On peripheral administration, A-317491 can access vascular compartments and thus peripheral tissues and imparts action at receptive field terminals for P2X3 containing neurons. It is possible that following such routes, the antagonist may also have the potential for action at sensory cell bodies in the ganglia (DRGs and cranial sensory ganglia), as well as other peripherally located cell types that may contain P2X3 (e.g., synoviocytes, bladder epithelium); little or no CNS penetration is likely, so central terminal P2X3 receptors are uninfluenced. On local administration (e.g., intraplantar or intravesical), high concentrations can be achieved in specific target receptive fields, but access beyond such sites (especially to ganglia or dorsal horn termini) will be negligible. After intrathecal delivery, activity would reflect antagonism of P2X3 containing receptors on central projections of afferent neurons within dorsal horn laminae and central brainstem projections of cranial sensory neurons. In an excellent overview, McGaraughty and Jarvis [49] described that A-317491 produced antihyperalgesic effects to mechanical and thermal stimuli, with greatest effect after systemic application, with local (intraplantar) and central (intrathecal) delivery producing smaller and less consistent effects. Thus, antihyperalgesic effect can arise at multiple points on the primary relay, and penetration of blood–brain barrier may increase effect magnitude.

A rodent model often employed for assessing potential for drug effect in osteoarthritis (OA) pain is based on intraarticular injection of monoiodoacetate (mIOA) into one knee joint of the rat. mIOA inhibits glyceraldehyde-3-phosphate dehydrogenase activity in chondrocytes, disrupts glycolysis, and leads to cell death [60]. Progressive loss of chondrocytes leads to histological changes of the articular cartilage over subsequent weeks that resemble the changes which occur in human OA, leading to joint discomfort exemplified by a shift in the weight distribution (asymmetry) to favor the unaffected limb. The selective and "drug-like" P2X3 antagonist AF-353 [61] has shown robust efficacy in this model of OA as well as in the classical adjuvant arthritis model of joint hyperalgesia, based on intraplantar CFA administration. In Fig. 3a, b, data are shown from a study where 7d prior intraplantar CFA led to

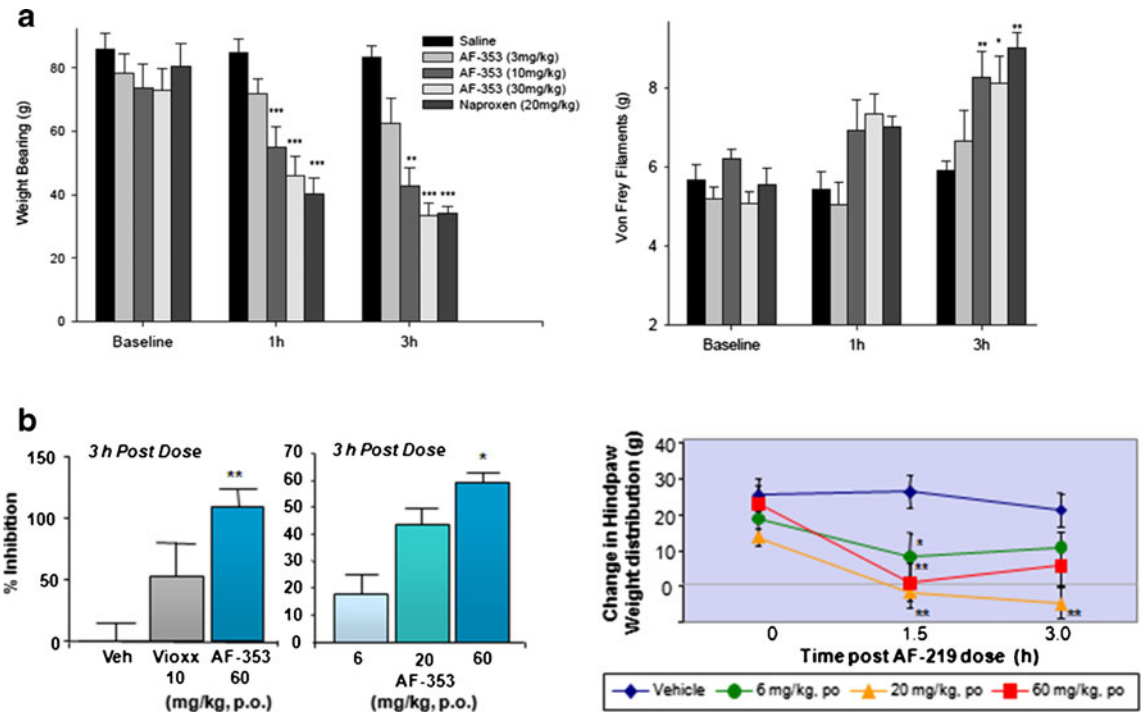

Fig. 3 Nocifensive data showing the effect of a P2X3 antagonist in preclinical models of joint hyperalgesia. **a** In an adjuvant-induced arthritis model in rat (7d following intraplantar administration of complete Freund's adjuvant), AF-353 produces dose-dependent anti-hyperalgesia in weight-bearing asymmetry and von Frey filament mechanical tests; magnitude of effect is compared with that of the NSAID naproxen. **b** In a rat model of knee osteoarthritis (14d following intra-articular administration of monoiodoacetate), AF-353 produces dose-dependent anti-hyperalgesia in weight-bearing asymmetry test (left; compared with the COX-2 inhibitor Vioxx). In the same model, following repeated dosing, AF-219 (7d bid, orally; right) attenuates the weight bearing laterality with complete reversal of apparent hyperalgesia at the two higher doses

mechanical hyperalgesia against which AF-353 produced dose-dependent reversal in both weight-bearing and von Frey filament tests of hyperalgesia (equivalent efficacy compared with the NSAID naproxen). In Fig. 3c, d, (Knee mIOA model, 14d prior intra-articular mIOA) acute oral administration of AF-353 produced dose-dependent anti-hyperalgesia in weight-bearing asymmetry (higher efficacy compared with a clinical relevant dose of rofecoxib), while the closely related antagonist AF-219 (after 7d bid, po dosing at 6, 20, and 60 mg/kg) fully reversed weight bearing laterality at 1.5 and 3.0 h after the final dose.

Most recently, reports emerged from rodent models associated with significant cancer cell invasion, usually to bone or cutaneous sites, modeled as surrogates for bone or soft tissue metastasis pain, wherein lytic cancer cells are carefully implanted into the large bones such as tibia or soft tissues such as gingiva. The erosion and inflammation causes significant change in sensory innervation to the affected area [62, 63] with P2X3 expression and function increased within these receptive fields and in adjacent overlying tissues [64, 65]. Consistent with this, antagonism of P2X3 receptors (using AF-353 or A-317491) has been shown independently by at least two groups to acutely reverse and chronically delay progression of development of tactile allodynia/hyperalgesia and/or raise weight-bearing thresholds [66, 67]. Figure 4 illustrates such data from a tibial invasion model, with significant effect produced by AF-353, given orally (twice daily), when given either during (preventative) or following (reversal) development of hyperalgesia.

While the relative contribution of central versus peripheral terminal P2X3 antagonism has been somewhat addressed in inflammatory and urological models, one aspect that has not been well addressed is whether the effects seen with various antagonists requires blockade of P2X3 homotrimers alone or P2X2/3 heterotrimers in addition. From studies using TNP-ATP, A-317941, AF-353, it is not easy to answer this question, as the antagonists block homo and heterotrimeric P2X3 receptors, albeit with slightly higher concentrations needed for the latter. In a recent report [68], scientists at AstraZeneca described findings with a novel antagonist (AZ004, a pyrrolopyrimidinone) which shows apparently high potency for inhibition of rat and human P2X3 (IC_{50} values 13–30 nM) while showing no activity at P2X2/3 heterotrimers at up to 2 μM (rat) or 10 μM (human). After subcutaneous administration, AZ004 was able to produce potent inhibition of paw mechanical hyperalgesia in rats pretreated with intraplantar CFA, which was reproduced by direct intraplantar, but not intrathecal injection of the antagonist. It remains open for confirmation whether this significant impact via peripheral P2X3 homotrimer blockade is unique to this particular model system or relatively general to these types of inflammatory models.

Collectively, while recognizing that any attempt to accept data from an animal model as predictive for human disease and discomfort is rash, the weight of evidence from a wide range of irritative and inflammatory models of musculoskeletal hyperalgesia make pretty reasonable the proposition that P2X3 antagonists should be assessed in chronic conditions such as in OA and RA, joint and muscle contusions and sprain, and even in chronic and breakthrough pain in patients with cancer.

Visceral pain

ATP regulation of sensory perception and pain in visceral structures has been described for a variety of organ systems, and forms a key component of Burnstock's unifying purinergic proposition relating to mechanosensation and control of movement and secretion in hollow organs (the "tubes and sacs" hypothesis; [69, 70]). It is suggested and with significant supportive evidence, that ATP, released from epithelial cells lining the bladder (Fig. 5), ureter, and gut during distension (though likely also from other cell types including smooth muscle), acts on P2X3 and/or P2X2/3 receptors on lumbosacral (pelvic) and thoracolumbar (splanchnic) subepithelial sensory nerve terminals to relay messages about stretch, pressure, irritation via sensory ganglia and spinal cord to pain centers in the CNS [70, 71]. Under chronic conditions of inflammation or injury, sensitization of these afferent pathways accompanies upregulation of expression of these receptors. In animal models and clinical investigations, evidence has also surfaced showing concomitantly increased local ATP concentrations, and P2X3 expression is also elevated in specimens from clinical bladder and gut pathologies [72–74].

Afferents fibers chemically traced from bladder, ureter, bowel, or esophagus show P2X3 expression and sensitivity to activation by ATP in high proportions (often >50%; [70, 71, 75]). Among these organs systems, the greatest attention has been placed on urinary tract, the role of ATP/P2X3 within the urinary sensory limb, and its contribution to storage and elimination reflexes in normal conditions and in disease. P2X3 and P2X2 subunit gene deletion in mice (either as single knock-outs or cross-bred double-KO mice) produced alteration in bladder reflexes (hyporeflexia) with considerable elevation of bladder volume thresholds [38, 39]) under conditions that expose C-fiber pathways. Greatly reduced firing from isolated bladder and ureter afferents in response to distension was also seen in these KO mice [76–78].

Selective P2X3 antagonists do lower urinary overactivity in disease models, the earliest such report examining the

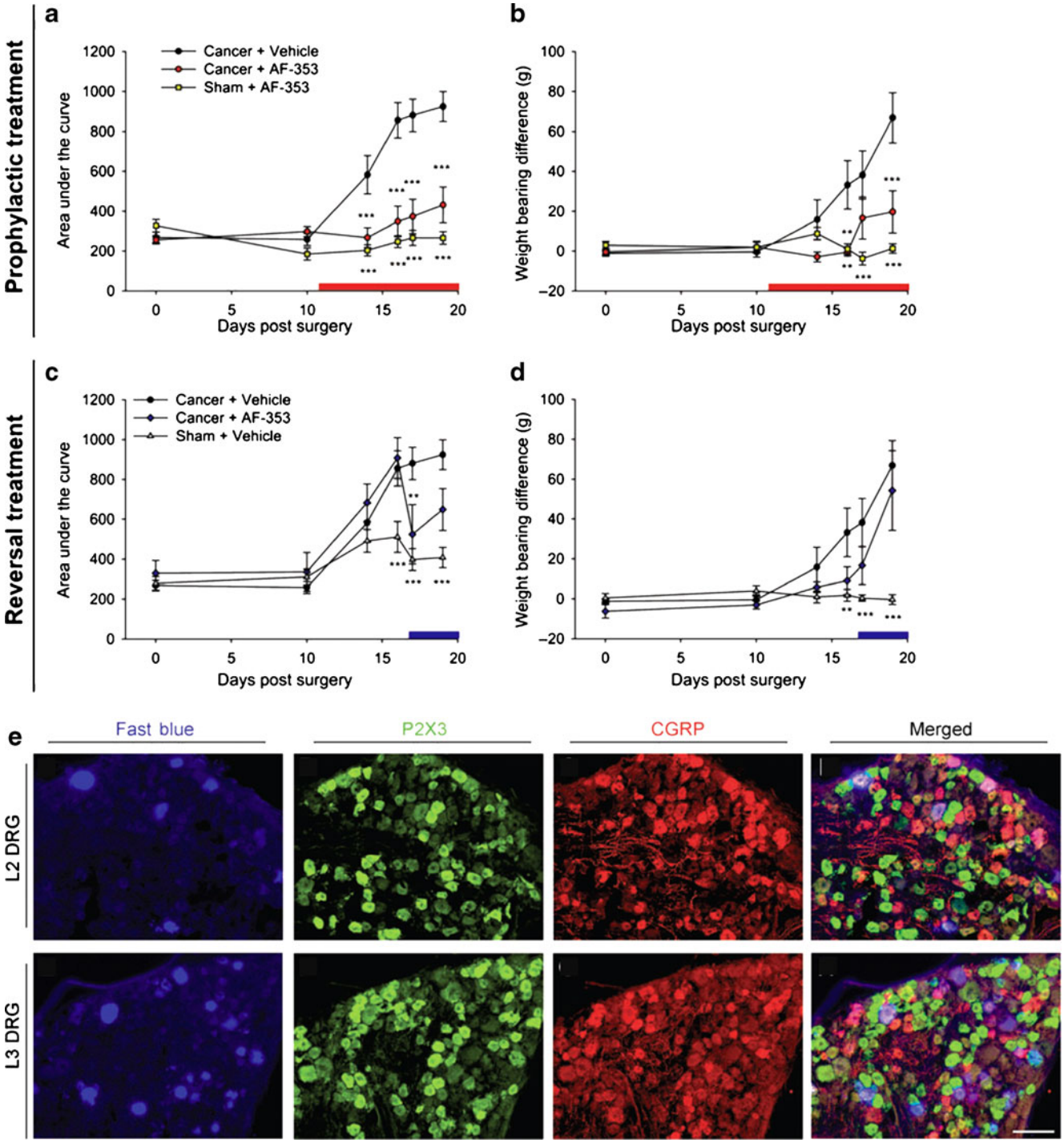

Fig. 4 Systemic P2X3 receptor antagonism with AF-353 attenuates bone cancer pain behaviour in rats. Intra-tibial injection of MRMT-1 carcinoma cells (*black circle*) induced significant increases in both mechanical allodynia (**a** and **c**) and weight-bearing difference (**b** and **d**) compared with the control rats (*yellow square* and *open triangle*). Oral administration of AF-353 (bid) significantly attenuated both MRMT-1 carcinoma cell-induced mechanical allodynia and weight-bearing difference when given before development of bone cancer pain behavior (termed prophylactic treatment, *red circle*), while bone cancer-induced established mechanical allodynia could be significantly reduced by AF-353 (termed reversal treatment: *diamond*). The *red* and *blue bars* represent the prophylactic and reversal treatment dosing periods, respectively. Mechanical allodynia was quantified by calculating the area under the curve values determined from a plot of the percentage of positive ipsilateral withdrawal response at each filament against the filament force on a logarithmic scale. Hindlimb weight-bearing difference was expressed as contralateral minus ipsilateral readings. Values are expressed as means± SEM. $^{**}P<0.01$ and $^{***}P<0.001$ versus the cancer+vehicle group (from [66]). In the lower panel (**e**) is shown immunohistochemical characterization of the retrogradely labeled Fast Blue DRG neurons innervating the rat tibia. Fast Blue (*blue*) labeling was present in both P2X3- (*green*) and calcitonin gene-related peptide (CGRP)-positive (*red*) neurons at the L2 and L3 DRG levels

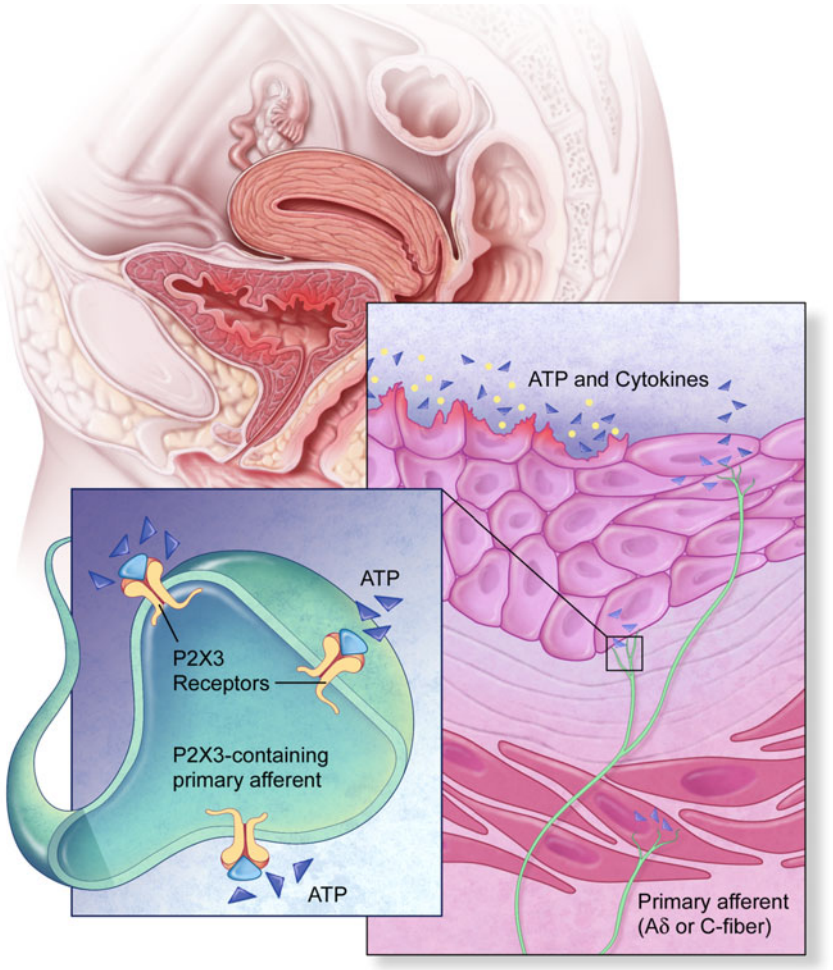

Fig. 5 Sensory fibers in visceral organs, especially the urinary bladder, express high levels of P2X3 receptors that are elevated in pathological conditions; unmasking segmental spinal reflexes that sense ATP content during filling and distension. P2X3 antagonists suppress afferent excitation and raise filling volume thresholds, especially in rodent models of cystitis. The distressing and largely unmet painful and irritative symptoms of bladder pain syndrome/interstitial cystitis and chronic prostatitis as well as lower urinary tract symptoms (urgency, frequency, and nocturia) associated with overactive bladder and benign prostatic hyperplasia, represent important visceral indications for novel P2X3 antagonists

effects of intravenous administration of A-317491 in a rat spinal cord injury model [78–81]. The antagonist produced a dose-dependent inhibition of non-micturition bladder contractions, increased inter-micturition interval, and bladder capacity, without influencing the amplitude of voiding contractions. This pattern of effects may seem fairly desirable if translated into a clinical setting, where inappropriate sensitivity to low volume filling, with exaggerated urinary frequency and sense of urgency could be suppressed while leaving untouched the force of detrusor contraction needed to maintain voiding efficiency. The relevance of the spinal transection model for human disease can be debated, but one of the purported consequences is that descending inhibitory pontine control of voiding reflexes is severed and so local segmental ("reflex bladder") C-fiber driven circuits become unmasked. This model is also associated with increased ATP liberation from bladder epithelial cells and increased levels of ATP in the dorsal horn of the lumbosacral spinal cord [82–84]. A-317491 was also studied in a cystometric model in rats pretreated (2 days prior) with cyclophosphamide [85] to produce an inflammatory urinary hyperreflexia. Similar responses were seen as in the spinal model, with increased intervals between voids, reduced non-micturition bladder contractions and amelioration of cyclophosphamide-induced residual volumes, all without impact on the amplitude of voiding contractions.

The diaminopyrimidine AF-353 was studied in a closed cystometric model ("refill VIBC") in a urethane-anesthetized rat with a dose-dependent increase in volume threshold by up to 50–70%, with frequency slightly reduced, but no appreciable change in amplitude (see Fig. 6a; [86]). As AF-353 penetrates the blood–brain barrier [61], it is not clear whether these effects resulted from P2X3 antagonism at peripheral terminals within the bladder wall, or alternatively at central terminals in the spinal cord dorsal horn, where is has been shown that ATP is released and can act on P2X3 receptors to enhance central glutamate transmission [18, 83, 87]. Two separate studies using distinct members of the diaminopyrimidine class helped to address the question of central versus peripheral mode [86, 88]) and it was seen that presynaptic

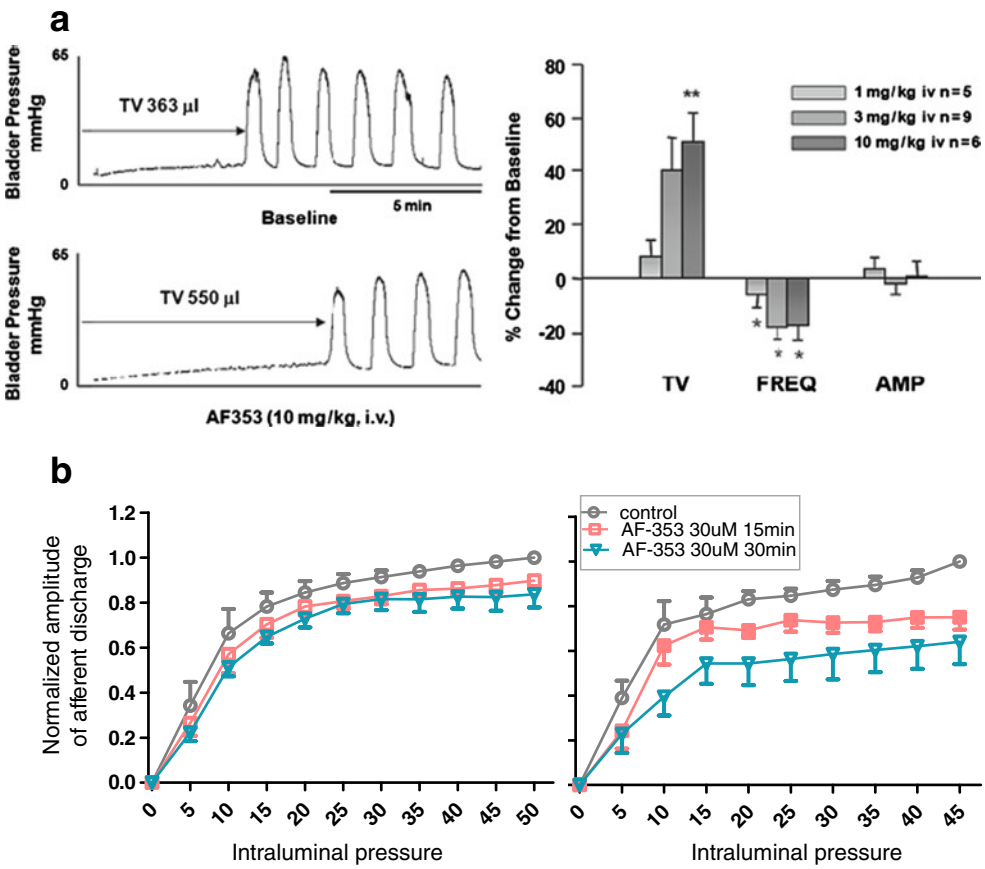

Fig. 6 Effects of P2X3 antagonists in preclinical models of urinary bladder reflexes. **a** P2X3 antagonism (AF-353) significantly raises volume thresholds and lowers frequency of micturition reflexes in filling cystometry models in rats anesthetized with urethane, which unmasks segmental spinal pathways. **b** Bladder–pelvic nerve preparation from cyclophosphamide (CPM) sensitized rats. Afferent nerve firing associated with volume expansion is markedly reduced by P2X3 antagonism with AF-353, applied either to serosal (extraluminal, *left*) or intravesical (intraluminal *right*) surface of the bladder (figures kindly provided by Prof Weifang Rong, Shanghai Jiaotong University, China)

P2X3 and/or P2X2/3 receptors on the central terminals of bladder primary afferents were able to facilitate both normal and noxious input from the micturition reflex. In Fig. 6b, recording of action potential discharge from pelvic afferents in an isolated bladder model from cyclophosphamide treated rats is shown, in which AF-353 applied either to the extraluminal or intravesical fluid is able to markedly suppress activity in response to filling. Whether the potential for therapeutic impact in urinary tract disorders such as overactive bladder (OAB), benign prostatic hyperplasia, lower urinary tract symptoms (LUTS), bladder pain syndrome (BPS)/interstitial cystitis (IC), or renal/ureteric colic requires blockade of peripheral, central, or all P2X3-containing receptors will be a matter for further exploration.

Nevertheless, this range of activities seen in urological models offers a reasonable foundation for P2X3 antagonist investigation in clinical conditions associated with LUTS and pain (e.g., OAB, BPS, chronic prostatitis). Generalization of these mechanistic findings across other hollow organs has been reported [89–91] and accordingly invites consideration of antagonist utility in functional gut disorders such as irritable bowel syndrome; it seems also clear that cross-sensitization (viscero-visceral and viscero-somatic) is an important topic in pain syndromes and this may become a valuable focus [92, 93].

Neuropathic pain

The evidence for P2X3 contribution to development of neuropathic pain in models was not initially considered strong, based on protein expression data, but over the last decade it has grown markedly especially on the basis of functional animal data examining modulation of developed hyperalgesia and allodynia. Bradbury et al. [16] had shown that P2X3 receptors in DRG and dorsal horn are down-regulated following peripheral nerve injury (sciatic axotomy) but that their expression can be up-regulated by concurrent glial-derived neurotrophic factor (GDNF) exposure. However, Eriksson et al. [94] demonstrated that ligation/section or chronic constriction of the mandibular inferior alveolar nerve led to significant upregulation of P2X3 immunoreactivity in trigeminal ganglia and in associated nerve endings. Similarly, Novakovic et al. [95] studied P2X3 purinoceptor cellular distribution in rat sensory ganglia and dorsal horn in naive animals and following peripheral nerve injury (sciatic chronic constriction injury (CCI) model) using immunohistochemical methods. Two to four weeks following unilateral CCI, the number of P2X3 positive small and medium diameter neurons increased in DRG, and expression levels were higher in spinal cord on the side ipsilateral to the ligated

nerve, consistent with up-regulation of receptors in presynaptic terminals of the primary sensory neurons. Since then, several reports have surfaced that essentially corroborate the findings of Novakovic et al. [95] in CCI and ganglion compression models of sciatic and trigeminal nerves [52, 96–100], and a single report has examined a clinical neuropathic pain condition [101] and shown upregulation of P2X3 neuronal expression.

These findings differed not only from those reported by Bradbury et al. [16] but also by Kage et al. [102] where following L5/L6 spinal nerve ligation the proportion of small-diameter neurons expressing P2X3 was reduced, although P2X3 immunoreactivity of medium-sized neurons in the L5 and L6 DRG was unchanged. Other recent studies have also shown either a down-regulation of P2X3 in the plantar dermal receptive field following sciatic CCI [103], a transient reduction in trigeminal P2X3 expression in model of partial injury of the mental nerve [104] or no change in P2X3 expression following lingual nerve section [105]. Exactly why this varied picture developed is not very clear, but it is possible that these discrepancies reflected a distinction in upward regulation of P2X3 expression following a constriction injury (loosely applied ligature and therefore non-axotomic) compared with reduction or no change following more extensive nerve injury or complete axotomy, where receptor loss may be expected. Thus, a key factor would be to differentially examine P2X3 expression in neurons that remain intact in axons or ganglia after traumatic intervention. Such an investigation was performed in one study [106], though looking at P2X3 mRNA rather than protein in DRG and trigeminal ganglia following tibial plus common peroneal transection or infraorbital nerve transection, respectively. These authors found that P2X3 mRNA in ATF3-immunoreactive neurons decreased significantly after injury, indicating that axotomized neurons lost P2X3 mRNA, despite an *overall* increase in P2X3 mRNA relative to the total number of sensory ganglion neurons. It was suggested that P2X3 mRNA expression increases in intact neurons and that P2X3 mRNA in intact neurons may contribute to the sensory changes post nerve-injury in primary sensory neurons. Similar observations were made in a recent report [104] in rat skin sensory nerves following partial loose ligation injury of the mental nerve, a purely sensory branch of the trigeminal nerve that innervates the lip. Most importantly, these authors tracked P2X3 expression in dermal and epidermal neurons following injury at multiple time points, and showed that a transient decrease in expression occurred over 2 weeks, followed by increases in subsequent weeks, which followed expression changes in GDNF in the skin, and also tracked with changes in markers of sympathetic and parasympathetic axon sprouting in the same receptive areas. Thus, timing of assessment of receptor regulation is critical and could account for mixed observations reported.

Despite a varied picture in protein expression studies in neuropathic models, a more consistent and positive picture has emerged in functional investigations of neuropathic hyperalgesia. Thus, using antisense and RNAi knockdown probes to suppress P2X3 expression following nerve injury [34, 41, 43], sensitization to tactile and thermal stimulation was reduced. Likewise, in several pharmacological studies in neuropathic pain models, reports have indicated increased sensitivity to ATP and analogs in sensitized neurons [52, 100, 107–110] or that selective antagonists, including A-317491 and AF-353, can reverse central sensorineuronal wind-up [111] or hypersensitivity to tactile stimulation [36, 37, 49], with evidence for effect dependent on a combination of peripheral and central actions.

The reports above offer justification for investigation of P2X3 antagonism in clinical neuropathic conditions, such as post-herpetic neuralgia (PHN), diabetic peripheral neuropathy (DPN), trigeminal neuralgia (TGN) and radiculopathy, although the concerns over patient heterogeneity (especially in DPN), and the questionable "face-validity" of neuropathic pain models remain clear. Such trials have generated more than their fair share of study failure and make one pause for thought about the best approach to clinical assessment.

Airways hyperreactivity

P2X3-containing receptors are expressed by a large proportion of C- and Aδ afferents that innervate the upper and lower airways arising from both placodal (nodose) and neural crest (jugular) ganglia [112, 113]. Earlier data on airways P2X3 had been mostly immunohistochemical (e.g., [114]), but more recently, data-driven arguments have surfaced [115] in favor of the proposition that afferent sensitization may drive a significant a component of key airway dysfunctions, signs, and symptoms (e.g., bronchial hyperreactivity, hypersecretion, cough, dyspnea; Fig. 7), with more attention placed on the possible role of P2X3 receptors. There are now several relevant reports that indicate P2X3 antagonism as an approach to reducing such airways sensitization.

In the primary model of cough used preclinically, in which aerosolized citric acid induced cough in guinea pig, it has been shown that ATP enhances citric acid cough, and that available P2X receptor antagonists block this effect in a manner consistent with involvement of P2X3 containing receptors [116]. In the same model, cough evoked by citric acid is also augmented by co-application with histamine, and this effect can also be significantly inhibited by P2X receptor antagonism, consistent with the effect of histamine being mediated via release of ATP within the lungs [117].

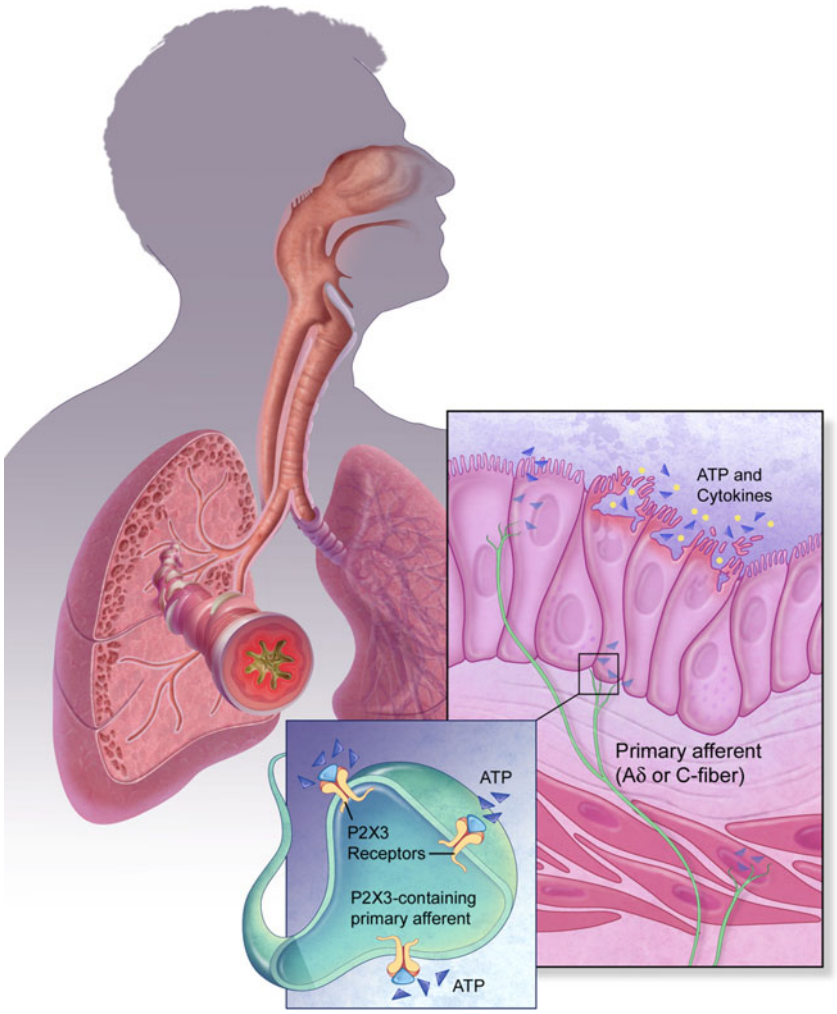

Fig. 7 Airway sensitization and irritation. Large proportions of C and Aδ fiber afferents in the upper and lower airways express P2X3 receptors and can be sensitized by ATP. Activation of some of these sensory nerves by ATP liberated from epithelial and smooth muscle cells is postulated to contribute to components of airways hyperexcitability, giving rise to airways hypersecretion, bronchospasm, breathlessness (dyspnea), and chronic cough, all of which are undermanaged factors in chronic diseases such as COPD and asthma

The characterization of the specific P2X receptor(s) involved is a little ambiguous, hampered by lack of specific probes, but is not inconsistent with P2X3 receptor involvement.

It is now established that P2X3 containing receptors are significantly involved in activation of vagal C-fibers and rapidly adapting receptors (Aδ-fibers) that are believed to be central to cough initiation and sensitization [113]. Using one of the first selective P2X3, P2X2/3 antagonists, the tricarboxylic acid A-317491 (Abbott), it has been shown that ATP activation of airways afferents was indeed mediated by P2X3 containing receptors [112]. Moreover, electrophysiological recordings from jugular and nodose afferents of guinea pig airways confirm that ATP acting via P2X2/3 heterotrimers in nodose fibers leads to action potential initiation [112]. More recently, an intermediary role for ATP linking bronchoconstriction with firing of airways sensory fibers has been mechanistically investigated, and P2X3 receptors (probably via P2X2/3 heterotrimers) clearly mediate bronchospasm induced afferent nerve activation in isolated perfused lung/vagus nerve preparations from guinea pig (Fig. 8; [118]).

In other investigations, it has been shown that extracellular ATP activates canine and rodent pulmonary vagal sensory fibres (Aδ and C), which then leads to neurogenic bronchoconstriction and cough [119]. ATP administered by aerosol inhalation in humans induces several airways effects including cough, bronchospasm, dyspnea, and tightness in asthmatics and effects are greater than seen in healthy controls, with significantly greater potency to ATP than for adenosine and AMP, the latter indicating that the effects are not primarily mediated via degradation products of ATP [120]. Additionally, it is reported that intravenous infusion of ATP in pre-terminal cancer patients gives rise to highly distressing symptoms of breathlessness, chest tightness, and dyspnea in a large number of subjects [121].

Finally, adding further support of the potential for ATP involvement promoting respiratory disease and dysfunction, it was recently shown that the concentration of ATP is

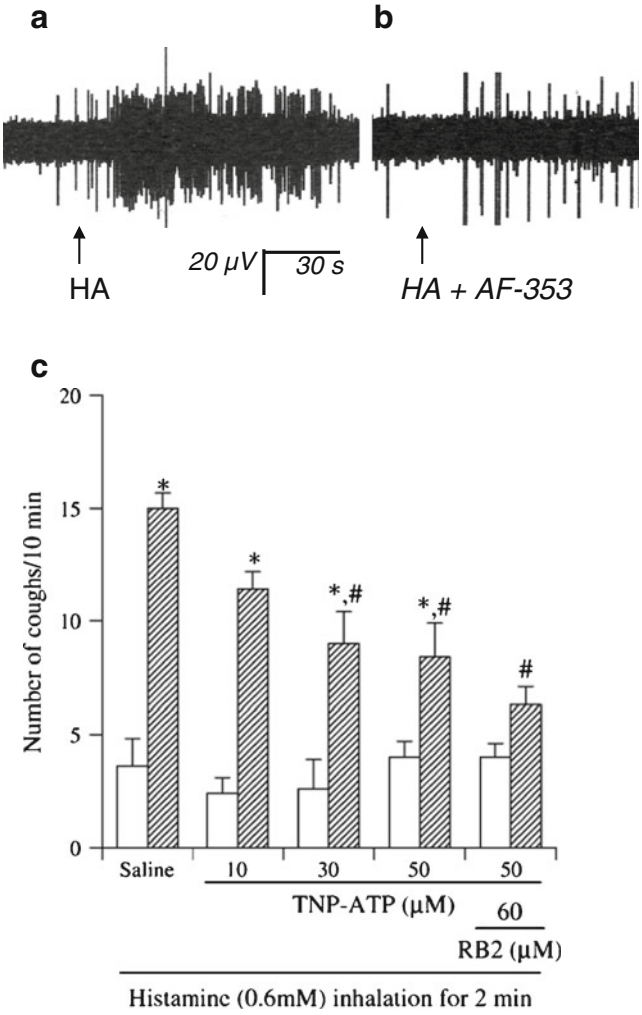

Fig. 8 **a**, **b** Extracellular recording of action potential discharge from an intrapulmonary nodose C-fiber in an ex vivo isolated, perfused lung-nerve preparation from guinea pig. **a** Representative recording of action potential discharge from a single lung nodose C-fiber (conduction velocity 0.78 m/s) in response to histamine (HA, 30 μM) delivered as a 1 ml bolus injection into both the pulmonary artery and the trachea. **b** Perfusion of the same C-fiber with AF-353 (100 μM, 15 min) reduced both the peak frequency of action potential discharge and the total number of action potentials (AP) generated in response to HA by 64% (figures kindly provided by Prof Brad Undem, Johns Hopkins University, USA). **c** Effect of TNP-ATP on the histamine-induced increase in the number of citric acid-induced coughs in guinea pigs. The guinea pigs were exposed to 0.6 mM histamine aerosol for 2 min during the 5 min preceding the inhalation of 0.1 M citric acid. Guinea pigs were exposed to TNP-ATP or reactive blue 2 (RB2) for 2 min during the 5 min preceding the inhalation of histamine. The number of coughs during 10 min of exposure to citric acid was counted before (open column) and after exposure to histamine (hatched column). Each column represents the mean with S.E.M. for five animals. *$P<0.05$ vs. the value before exposure to histamine. #$P<0.05$ vs. the value after exposure to histamine of saline-treated guinea pigs (reproduced with permission from [117])

markedly elevated in the bronchoalveolar lavage fluid from COPD patients, with smoking exacerbating the ATP elevations and concentrations showing marked correlation with increased obstructive measures and symptoms [122, 123]. In this study, it was concluded that "COPD is characterized by a strong and persistent upregulation of extracellular ATP in the airways" and although the authors' focus was on the contribution of extracellular ATP to the pathogenesis of COPD (by promoting inflammation and tissue degradation, purportedly through P2X7 activation), the likelihood that afferent sensitization induced by available ATP heightens symptomatic manifestations, morbidity, and mortality should not be overlooked. Therefore, the management of critical airways disease characteristics and symptoms of bronchospasm, hypersecretion, cough, and dyspnea that are very poorly met with existing therapy in chronic asthma and COPD represents a possible target for P2X3 blockade.

P2X3 antagonists: additional therapeutic avenues, safety, and tolerability

The sections above cover areas where the supportive data for P2X3 antagonism have been most abundant. Clearly, the need now is for focused clinical studies, as animal models, useful as they are, are far from optimal representations of clinical syndromes and distressing symptoms. What seems apparent considering the rationales detailed above is that P2X3 antagonism has a uniquely broad range of activities across visceral, inflammatory and neuropathic models, whereas most of the major analgesics are less broadly active across models.

There are other sensory systems that are worthy of mention, studied in the context of P2X3 activity, though in many cases without the benefit of selective inhibitors, and relying more heavily on gene or protein expression data or studies using non-specific probes. These reports offer bases for additional rationales for P2X3 antagonist potential; likewise, it also has to be considered that some activities of P2X3 containing receptors may contribute to important physiological functions, and that their antagonism may lead to clinically significant adverse events that affect tolerability or safety in some patient groups, and that may weaken the potential clinical effectiveness of the class overall. In the following section, such considerations are discussed.

In the largest sensory organ, the skin, it is quite apparent that the expression levels of P2X3 receptors (possibly more often as homotrimers) and levels of C-fiber activation by ATP are high [75] and represented on several distinct subgroups of fibers. A specific population of so-called tactile C-fibers has been identified in rodents that express a particular subtype of the Mrgpr receptor family (mas-

related G-protein coupled receptors; also previously reported as sensory neuron-specific receptors, SNSR; see [124, 125]: these are the MrgprD C-fibers (or MrgD fibers), which unlike the MrgA, B or C groups, are subject to *persistent* expression of the runt domain transcription factor, Runx1 [126]. MrgD afferents make up ~60% of the epidermal innervation [125] and in particular innervate the outermost of the four layers of epidermis, the stratum granulosum. Notably, classical C-fiber sensitizers such as capsaicin, serotonin, protons, nicotine, menthol, peptides (CGRP, substance P) do not activate MrgD fibers, but they are sensitive to ATP, and respond with kinetics that are consistent with the P2X3 homotrimer [127]. The implication is that these afferents, sitting closest to the external environment, are sensitive to ATP presumably released by mechanical activation of keratinocytes and brushing of hair fibers, though it is certainly not yet clear whether these are nociceptors or perhaps lower threshold mechanosensors, or function with broad dynamic range contingent on trophic factor interplay.

It remains unclear whether human cutaneous afferents are similarly organized, though sensitivity to ATP has been clearly shown to injection, iontophoresis and blister application (as discussed above). It is possible that MrgD afferents correspond to the so-called C-tactile fibers that are found only on hairy skin and are responsive to gentle stroking evoke pleasurable or "hedonic" tactile sensations [128, 129]. Antagonism here may remove a potentially pleasurable social response, unlikely though this would be a major issue to drug tolerability. On the other hand, it should be considered that in chronic painful conditions, especially neuropathic injuries such as in shingles, post-herpetic and trigeminal neuralgia, and in more subchronic hypersensitivies such as following burn injury [130, 131] or even sunburn [28, 132], the phenotypic sensory change is such that mild tactile stimulation and stroking can now be perceived as highly painful in the affected dermatome, and accordingly there may be significant anti-allodynic benefit to P2X3 antagonism, with the potential even for topical application. A further potential area for consideration, though again inadequately investigated, is the potential that hypersensitivity in these afferent populations may have any bearing on conditions associated with persistent and troublesome itch (or indeed the perceived benefit and transient relief of a good itch-induced scratch), though in the context of these C-fiber populations, there has been no evidence presented.

Just as ATP and P2X3 receptors seems to be a key part of the primary mechanical sensing system in the superficial layers of the skin, ATP signaling also occurs in the special senses, participating in the perception of physical and chemical signals, although with it being less clear in many cases whether P2X3 subunit containing channels are so intimately involved (reviewed by Housley et al. [133]). In the visual system, there is clearly immunohistochemical presence and functional involvement of most ATP receptors in many cells and functions, but only in a couple of reports has any evidence been apparent for expression of P2X3 subunits in retinal ganglion cells (among most other P2X subunits; [134, 135]) and no functional evidence is apparent. In the olfactory system, it has been established that both P2Y and P2X receptor activation by ATP reduces odor responsiveness [136]. Non-selective purinoceptor antagonists increase the odor-evoked calcium transient, providing evidence that endogenous ATP down-modulates odor sensitivity via activation of multiple subtypes in olfactory receptor neurons; however, the distribution of P2X3 subunits is limited in olfactory systems, and thus selective antagonists are unlikely to impart much impact on sense of smell based on the evidence to date [133].

In the auditory system, there is somewhat greater evidence for the expression and function of P2X receptors [133], though the major role of ATP in modulating hearing seems mediated via P2X2 homotrimers. Purinergic signaling appears to be involved in the regulation of hearing sensitivity especially under conditions of stress, acting via the P2X2 subtype receptor in tissues lining the endolymphatic compartment [133, 137]. ATP may be a neuromodulator at the primary synapse between the inner hair cell and auditory afferent neurons and a neurotransmitter at the type II synapse with the outer hair cells which regulates the generation of spontaneous neural activity in the developing cochlea, though again any pivotal involvement of P2X3 subunits has not been shown. ATP may be involved in neurite growth in primary afferent neurons and synaptogenesis of the primary synapses, with action via P2X3 containing receptors, the expression of which follows development and maturation of the synapse in the rat and mouse [138–140]. It is not clear whether these possible actions of ATP at P2X3 containing receptors would manifest noticeable changes to hearing as a result of selective antagonism or whether any potential for benefit could arise.

In the gustatory system, ATP undoubtedly plays a role in signaling between most oral and post-oral taste buds and the gustatory C-fibers that innervate them. Studies using P2X gene deletion mice developed at Roche [38, 39] have illustrated that absence of *both* P2X2 and P2X3 subunits (double KO mice) almost abolishes taste transmission (in the chorda tympani and glossopharyngeal nerves) and discriminatory behavior, examining sweet, bitter, salty, sour and umami tastants [141, 142]. The near abolition of tastant sensitivity in the double KO mice was only marginally manifested however, when looking at gustatory nerve recordings or discriminative two-bottle preference tests in single P2X3 or P2X2 KO mice, suggesting that channel

redundancy operates in the transduction of taste bud responses. Subsequently, the phenotype of the P2X2 and P2X3 double KO mice has been elucidated further, and it now appears that the near complete loss of gustatory function occurred not solely due the absence of C-afferent ATP receptors, as the pannexin channel release of ATP from taste buds themselves was eliminated in the double [143] but not single KO mice. As P2X2 but not P2X3 receptor subunits are present on taste buds, it seems that the elimination of P2X3 subunits may blunt but is unlikely to totally impair taste perception. As antagonism of P2X3 and P2X2/3 trimers will not impair function at homomeric P2X2 receptors on both taste buds and gustatory C-afferents, the net impact of antagonism is difficult to predict, and has not been studied using selective antagonists in rodent models. In fact, earlier pharmacological exploration in rat isolated tongue–nerve preparations [144] had indicated limited impact on tastant sensitivity.

In the craniofacial circuitry, P2X3 expression and responses are seen in cranial afferents, particularly relating to the trigeminal ganglia and innervation of dental pulp, skin, muscle (e.g., masseter) and temporo-mandibular joint (TMJ), as well as dural afferents with relevance to headache and migraine. P2X3 receptors are fairly prominently expressed (30–60% of small- and medium-sized afferents), and are upregulated following nerve injury or inflammation in essentially all of these receptive fields, both in small fibers that are labeled by the isolectin IB4 as well as medium-sized afferents not bound by this probe [145–152]. Fewer pharmacological studies have been undertaken, though findings have implicated P2X3 and P2X2/3 receptor activation in trigeminal neuralgia, TMJ syndrome, burning mouth syndrome, dental pain and migraine. In the particular case of migraine, significant interplay between nerve growth factor, substance P, CGRP and P2X3 has been detailed [153–156], which may indicate a degree of regulatory differentiation from DRG circuits where the functional overlap of P2X3 and peptide containing afferents seems more limited. Clearly, this is an area which is worthy of additional focus to understand whether P2X3 antagonists might have some applicability in craniofacial pain syndromes or even headache, where unmet need persists and options are sparse.

Another area of emerging interest in the P2X3 field cover the sensory responses to ischemia in various tissues and ischemia-associated reflexes that may be evoked or altered in cardiovascular or skeletal circuits. There is a basis to suspect that ATP is released in fairly copious amounts from cells subjected to acute or chronic conditions of ischemia [157–162], and this has been shown in the context of ischemia of cardiovascular tissue as well as skeletal muscle. In the context of cardiac and vascular reflexes, Xu et al. [163] reported that in the dog, ATP triggers a cardio-cardiac vagal depressor reflex by activating presumed P2X2/3 receptors (based on sensitivity to antagonism by TNP-ATP, and lack of desensitization) found on vagal sensory terminals in the left ventricle. These authors suggested that these reflexes may contribute to vasovagal syncope, intimating a role for extracellular ATP in this syndrome and that endogenous ATP released from ischemic cardiomyocytes may be a mediator of atropine-sensitive bradyarrhythmias associated with left ventricular myocardial infarction.

Stellate ganglion (SG; sympathetic) cardiac afferents were studied in a rat myocardial ischemia model [164], and P2X3 labeling intensity and the P2X3 fiber proportion in SG were enhanced, suggesting the involvement of P2X3 receptors in sensory transmission after myocardial ischemic injury. This has since been characterized with similar findings in parasympathetic nodose afferents [165]. This group subsequently confirmed that P2X3 receptors mediate ischemic nociceptive signaling by showing sensitivity of SG responses to A-317491 [166], and that blocking P2X3 receptors in this model in vivo led to reduced evidence of cardiac dysfunction, arrhythmia and injury [167] as well as antagonism of increased sympathoexcitatory reflexes [168]. Fu and Longhurst [169] have extended such observations of sympathetic cardiac afferents in rat, showing that during ischemia, endogenously released ATP activates ischemia-sensitive, but not ischemia-insensitive, cardiac spinal afferents through stimulation of P2 receptors likely located on the cardiac sensory neurites, though lacking conclusive evidence for P2X3 involvement. Developing this line of pursuit further, it has been reported recently [170] that cardiac spinal (sympathetic) afferents are excited by ischemic metabolites and elicit an excitatory sympathetic reflex which plays a major role in the genesis of ventricular arrhythmias. Subjecting rats to cardio-spinal deafferentation, disrupted this reflex offered protection against ischemia-induced ventricular arrhythmias, and was associated with a reduced cardiac metabolic demand (lower rate-pressure product and ST segment elevation) during brief myocardial ischemia.

The exercise pressor reflex (EPR) is a homeostatic reflex composed of a mechanoreflex and a metaboreflex in normal healthy individuals, and represents another afferent activated system that may impinge upon fibers that express P2X3 receptors, with evidence for greater contribution in disease [171]. During sustained skeletal muscle work, as well as in models where blood flow to skeletal muscles is impaired, an exercise pressor reflex is triggered. This important reflex is activated during contraction of muscle primarily by stimulation of "ergo-receptive" (as opposed to nociceptive) afferent fibers responsive to either mechanical distortion (so-called group 3 fibers) and/or the metabolic by-products of skeletal

muscle work (group 4 fibers). EPR is known to be accentuated in patients with cardiovascular disease (such as heart failure, hypertension) as it is in rats with muscle or cardiac ischemia, leading to inappropriately exaggerated reflex pressor and tachycardia responses, and poor tolerance of exercise [171–173]. It is postulated that heightened mechanoreflex (group 3) and attenuated metaboreflex (group 4) activity may underlie such changes [171]. ATP release [162], coupled with upregulation and activation of P2X3 receptors (probably in concert with other candidate "metaboreflex" receptors such as TRPv1, ASIC3) contributes to these exaggerated reflexes [174, 175] and P2X3 receptors may contribute to accentuation of the sensitivity of the group 3 (purportedly mechanoreceptive) fibers [176]. However, it is worth noting that some ischemic responses induced by ATP release from myocardial and skeletal muscle tissues are not P2X3 dependent; for example, the study by Birdsong et al. [161] elegantly described how ATP increased the pH sensitivity of acid-sensing ion channel 3 (ASIC3), the sensory receptor for lactic acidosis, via what appears to be a population of C-afferent P2X5 receptors. Furthermore, most data supporting P2X3 involvement in EPR stem from studies in decerebrate animals, and one may ask how functionally relevant this is for the EPR in intact animals let alone in healthy humans and heart failure patients. It will be also interesting to understand whether the chronic benefits of exercise to reduce EPR in ischemic rats [176] is itself associated with any change in expression or function of P2X3 receptors.

Taken together, this varied collection of reports from investigations of integrated cardiovascular reflexes indicates that ATP signaling is heightened as a consequence of fatigue and ischemia in skeletal and cardiac muscles, and that this is associated also with an increased expression and function of P2X receptors—including P2X3—on sympathetic and parasympathetic sensory neurons. It appears that this heightened sensitivity to ATP in ischemic disease may lead not only to increasing fatigue and declining performance, but potentially can exacerbate cardiovascular outcomes. In patients with heart failure, there is intolerance to exercise reflected by dyspnea, hyperventilation, increased sympathetic drive, and hypertension that in turn increase risk of stroke and lethal arrhythmias [177]. Given these P2X3 connections to pathological reflexes, coupled with the potentially deleterious role P2X3 activation may have in the airways directly (see above), selective antagonism of P2X3 containing receptors may offer utility in suppressing a variety of co-conspiring inappropriate reflexes and provide new therapeutic avenues in these conditions with significant morbidity and unmet need. An agent that can stifle the exaggerated EPR in patients with cardiorespiratory disease and suppress the exercise-limiting sequela of dyspnea could offer some unique benefits. Clearly, an important question that remains to be elucidated is the extent to which P2X3 dependent C-afferent pathways contribute to physiological reflexes versus the pathophysiological reflexes mentioned above.

Drug discovery achievements

Prior to 2000, there were no reports of "drug-like" small molecules that selectively antagonized the activation of P2X3-containing receptors by ATP. Existing antagonists were large polyanions (e.g., suramin, PPADS) with little specificity or nucleotides (TNP-ATP), neither of which provided ideal starting points for medicinal optimization (Fig. 9). More promising leads were then reported and patented by Abbott, including A-317491, which offered submicromolar potency, competitive and selective antagonism at P2X3 and P2X2/3 receptors, and with properties that could offer applicability for in vivo studies in sensory models. However, these compounds (tricarboxylic acids) were still polyanions, and despite good plasma half-life in vivo, A-317491 was almost completely protein bound and had essentially no permeability from enteric into systemic compartments or from systemic to central. Despite considerable effort, this class of competitive ATP antagonists could not be optimized chemically into suitably developable small molecule candidates as the removal of these highly acidic residues presumably led to loss of activity [61, 178].

The first patents and publications that suggested potential for drug-like chemistry emerged from Roche Pharmaceuticals from 2004 onwards. Several novel classes of compounds, operating non-competitively with ATP, were optimized for antagonistic potency at P2X3 and P2X2/3 receptors, elaborating chemotypes more compatible with medicinal optimization and revealing the presence of multiple binding sites, allotopic to the nucleotide binding site, at which selective inhibition could be imparted. Several distinct chemical scaffolds were identified with activity at one or more of these P2X3 receptor related sites, and thousands of compounds were directly synthesized to optimize within these classes. Many potentially developable medicinal candidates of high potency and selectivity with favorable physicochemical, pharmacokinetic, toxicological and in vivo pharmacological profiles have been generated. Likewise, several other companies identified potentially developable molecular scaffolds, some similar to as well as distinct from those identified at Roche, including Evotec (previously Renovis), Astra-Zeneca, Merck, and Shionogi (see Fig. 9 for several key chemical examples).

A brief insight into the chemistry related to two of the Roche chemical scaffolds - the diaminopyrimidines (DAPs) and substituted arylamides (SAAs), follows. In each case, the chemical starting point was identified by high-

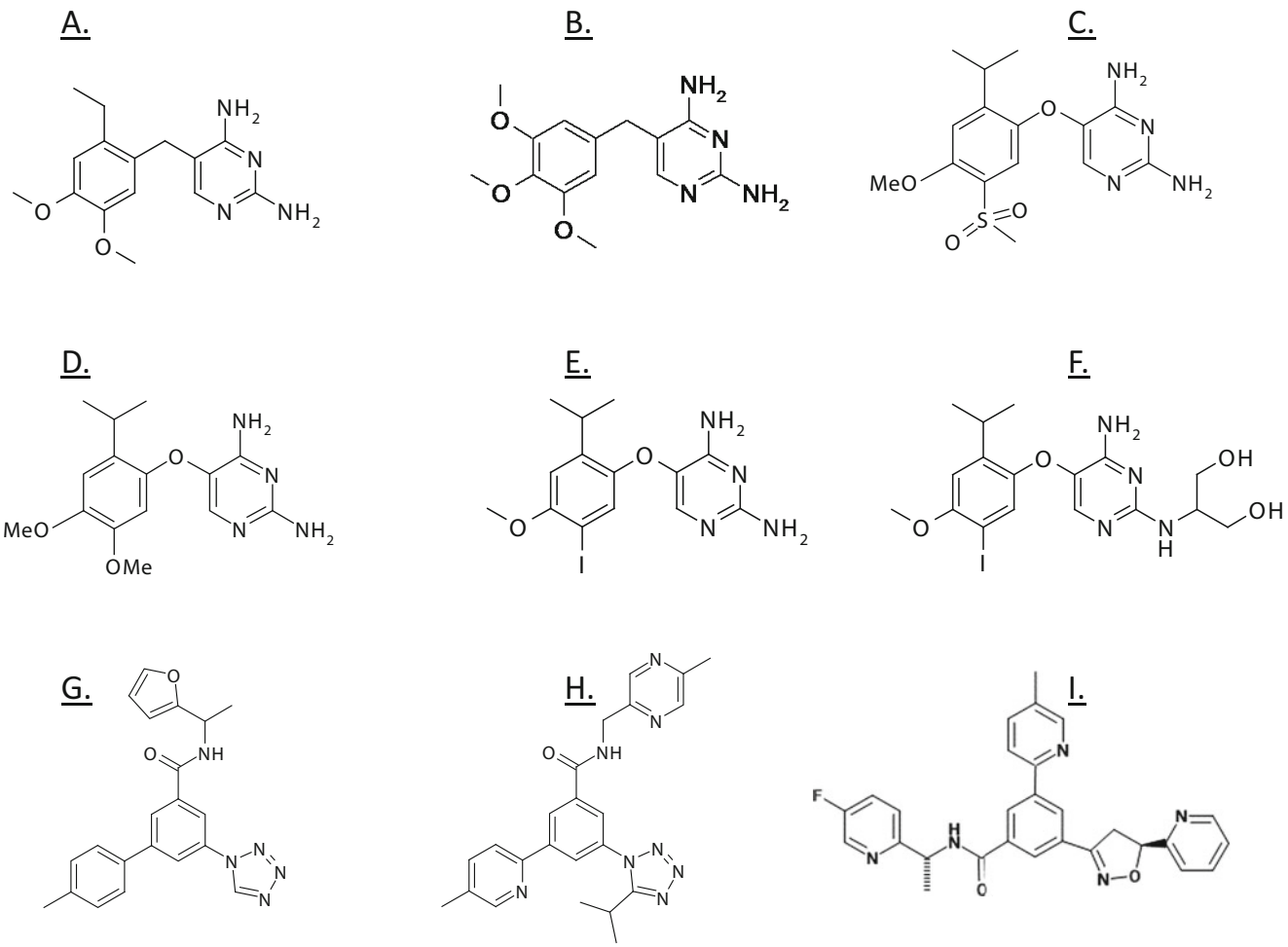

Fig. 9 Chemical structures of various key compounds that antagonize P2X3 containing receptors: **a** AF-001 (original DAP HTS "hit", P2X3 IC_{50} 1.4 μM); **b** trimethoprim (inactive at 30 μM); **c** AF-130 (RO-13; 100 nM); **d** AF-010 (RO-10; 40 nM); **e** AF-353 (RO-4; 6 nM); **f** AF-906 (RO-51; 2 nM); **g** AF-454 (original SAA HTS "hit", 0.65 μM) ; H, AF-014 (3 nM); I, MK-3901 (Merck lead published at ACS 2011; 24 nM)

throughput screening (HTS) of the vast Roche compound collection. Low affinity inhibitors (leads) were confirmed from a diverse (though very low percentage) number of compound hits from assays conducted at 10 μM at recombinant human P2X3 (DAPs) and P2X2/3 (SAAs) receptors. These assays made use of fluorometric imaging plate readers (FLIPR) running in 96 or 384 well capacity, screening ATP or α,β-methylene ATP (α,β-MeATP) activation of cells loaded with the Ca^{++} selective dye Fluo-3. Compounds were likewise profiled for selectivity by comparison of activity at P2X1, P2X2, P2X4, P2X5, and P2X7 receptors (at which all compounds tested were remarkably inactive), in addition to standard profiling in CEREP (GPCRs, ion channels, enzymes) and Ambit (kinases) batteries.

As lead optimization continued, most attractive candidates were tested for activity at native P2X3 containing channels, studying inhibition of ATP or α,β-MeATP excitation of isolated DRG or nodose ganglion cells by patch clamp electrophysiology, followed by assessment of effect in vivo in a range of models of nociception (acute, chronic, inflammatory, and neuropathic) and visceral sensitization. The most attractive leads were then assessed for in vitro and in vivo safety and PK desirability before advancing for chemical scale up and more thorough assessment for developability.

A) DAPs

The chemical starting point (a confirmed HTS "hit") for the DAP series came from a series of compounds generated at Hoffmann-La-Roche (Basel) in the early 70s to explore structure activity relationships (SAR) of congeners of the bacterial dihydrofolate reductase (DHFR) inhibitor trimethoprim, long used as an antibacterial. The hit DAP Compound had an IC_{50} of 1.2 μM at P2X3 receptors. Several close analogs that were also present within the

Roche collection as well as trimethoprim itself were seen to be completely inactive at 10 μM, providing early evidence of tight requirements in SAR.

Key advances in the SAR came from (a) adding the 2-isopropyl (though propyl, *t*-butyl, and methoxy were all inactive), (b) replacing the carbon linker between aryl and DAP groups with oxygen (where greater H-bonding reduced the available conformations) and, (c) substituting various subgroups on the aryl 4 and 5 positions to enhance potency and metabolic stability. A range of 5-substitued analogs were generated with varied physicochemical properties (particularly clogP and polar surface area) that were likely to impact permeability (from gastrointestinal tract and into CNS), metabolic clearance route, and potency, with several compounds developed as pharmacological tools as well as developable medicinal candidates. Of these, AF-010 (5-MeO), AF-130 (5-SO_2Me), AF-353 (5-I; previously RO-4) and AF-792 (5-acetylene) have been well described in literature [2, 61, 179–181]. The clinical lead compound, AF-219, is a very close analog of these and was advanced into development having best satisfied the required characteristics for development.

B) SAAs

The chemical starting point for the SAA series emerged from a FLIPR based library screen at the heterotrimeric P2X2/3 receptor, and was a unique compound in the Roche library (singleton), and turned out to be structurally related to a lead subsequently identified at Merck. Greater chemical latitude for optimization was apparent based on pharmacophore models within this chemotype that have since been iteratively developed, both at Roche [182, 183] and Merck [184], leading to many potent, selective, and biologically active antagonists that also represent candidates for medicinal development and have shown activity in a range of sensory models in vivo. Figures 9 shows examples of potent optimized lead molecules from the SAA chemical series, all displaying potencies in the 3–100-nM range at P2X3 containing receptors with no activity at concentrations below 10 μM at other P2X receptors, nor at a wide range of classical transmitter receptors, enzymes, channels, and kinases. The properties of a lead compound from Merck's SAA optimization were recently disclosed (ACS meeting, Anaheim, March 2011), MK3901 being described as a potent, selective CNS-penetrant P2X3 antagonist (IC_{50} ~23 nM) with activity in CFA, mIOA, and Chung (SNL) models of inflammatory and neuropathic pain.

Drug development

Afferent Pharmaceuticals, which licensed the P2X3 antagonist program from Roche in 2009, is currently advancing several compounds through early development phases. The clinical lead compound, AF-219, is a DAP series member close in structure to the 5-aryl substituted compounds shown in Fig. 9. AF-219 has completed IND-enabling GLP safety studies and three phase 1 studies in normal healthy volunteers. The compound is being developed as a twice daily, oral tablet, and has shown excellent safety and tolerability in clinical studies to date, with exposures achieved that significantly exceed those projected to fully engage the target and shown effective in preclinical rodent models, as well as showing activity in a clinical biomarker provocation assay. A series of phase 2 "POC" studies has now been initiated, with data anticipated in 2012 in several patient populations, designed to explore indications where blockade of P2X3 containing receptors may offer best clinical utility: these include joint pain (OA of the knee), visceral pain (BPS/IC), and airways sensitization (chronic idiopathic cough). Additional studies in other types of pain utility of will also be considered. This breadth of approach reflects (a) the wide range of tissues and clinical situations where ATP signaling has been reported to contribute to sensitization in chronic pathology through direct effect on sensory fibers, (b) the extensive evidence presented from rodent studies in KO mice (gene-deletion mutants), anti-sense/RNAi investigations as well as studies using small molecule probes, and, (c) the historical opportunities and threats observed in effectively translating novel sensory targeted interventions into the clinical realm.

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