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Systematic Review

A Systematic Review of the Expression, Signalling and Function of P2 Receptors in Primary Bone Cancer

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Abstract

Primary bone cancers are rare malignant diseases with significant morbidity and mortality. The treatment regimen relies on a combination of surgery (often involving amputation), chemotherapy and radiotherapy with outcomes dependent on localization of the tumour, grade, size and response to chemotherapy. Both treatment options and survival statistics have remained constant over the past 40 years and alternative therapies need to be explored. Purinergic signalling involving the interaction of extracellular nucleotides with P2 receptors has been investigated in numerous cancers with activation or inhibition a topic of debate. To assess whether purinergic signalling could be a viable target in primary bone cancer a systematic review for relevant primary literature published in PubMed, MEDLINE and Web of Science was performed. Search terms were formulated around three separate distinct topics; expression of P2 receptors in primary bone cancer models, P2 receptor signalling pathways involved and the functional consequences of P2 receptor signalling. Searching identified 30 primary articles after screening and eligibility assessments. This review highlights the diverse expression, signalling pathways and functional roles associated with different P2 receptors in primary bone cancers and provides a systematic summary of which P2 receptors are exciting targets to treat primary bone cancer and its associated symptoms.

Keywords: ATP; chondrosarcoma; chordoma; Ewing's sarcoma; osteosarcoma; P2 receptors; primary bone cancer; purinergic signalling; tumour microenvironment

1. Introduction

Primary bone cancer (PBC) refers to a heterogeneous group of distinct neoplasms affecting the skeleton arising directly in the bone [1]. They are comprised predominantly of osteosarcoma (OS), Ewing's sarcoma, chondrosarcoma and chordoma [2]. OS is the most common major subtype and is a malignant tumour mainly affecting children and young adults in the long bones of the extremities with two peak incidences between 10–19 [3] and >60 years [4]. It is characterised by bone formation and is highly heterogeneous, with an unknown aetiology [5]. Ewing's sarcoma is a malignant tumour that affects a similar age demographic to OS with a peak age of incidence of 15 years [6]. Ewing's sarcoma commonly affects the long bone of the extremities but are also abundant in the pelvis area and can also develop in soft tissues. Ewing's sarcoma is characterised by a distinct t (11;22) (q24;q12) chromosomal translocation encoding the EWS/FLI oncoprotein which is present in approximately 85% of cases [7]. Chondrosarcomas are malignant slow-growing tumours and involve the excessive production of neoplastic, cartilaginous tissue [8,9]. Chondrosarcoma, unlike OS and Ewing's sarcoma, is a disease predominantly seen in later adulthood [8,9] with a wide peak age of incidence between 30–60 years, they commonly occur in the pelvis, but can also arise in the femur or humerus [8]. Finally, chordomas are malignant slow-growing tumours that arise in the remaining notochord cells which are present during early axial skeletal development, preceding the backbone and skull base [10,11]. These areas are therefore the predominant site for chordoma which has a peak incidence in late adulthood between 75–84 years of age [11]. Both chondrosarcoma and chordoma have an unknown aetiology. Other bone tumours are not malignant and are benign, their incidence is debated as they can be asymptomatic and difficult to detect [12]. They are usually only discovered when examinations take place for other conditions. Although they are not as dangerous as malignant tumours, they can grow and compress the healthy surrounding tissue [13]. The most common benign bone tumours are osteochondroma and giant cell tumour of bone (GCTB) which can affect any age, however, are slightly more abundant in younger patients [13]. The survival statistics for PBC depend on a number of factors including the type, location, stage/grade and the presence or absence of metastasis. The statistics have remained constant for over 40 years with the 5 year survival rate for OS around 60% when localised, this decreases to around 20% with metastasis [14]; for Ewing's sarcoma the localised survival rate is 60–70% decreasing to 20–40% with metastasis [8]; for chondrosarcoma the 5 year survival is 75% [15] and for chordoma is 67% [16].

These statistics highlight the need to explore novel therapeutic strategies.

A potential novel therapeutic strategy is targeting purinergic signalling. Purinergic signalling involves the action of extracellular nucleotides such as ATP, ADP, UTP and UDP acting on P2 receptors [17], which are subdivided into P2Y and P2X subtypes [18]. There are eight P2Y receptor family members, which are all G protein coupled receptors with 7 hydrophobic transmembrane domains with three extracellular and three intracellular loops, an extracellular N terminus and intracellular C terminus [19]. These are then further subdivided based on their sequence and receptor G protein coupling, P2RY1, P2RY2, P2RY4, P2RY6 and P2RY11 couple to G_q and P2RY12, P2RY13 and P2RY14 coupled to $G_{i/o}$ [20]. There are 7 P2X receptor family members, which are ligand gated ion channels that respond solely to ATP and ATP analogues and range in size from 388 to 595 amino acids [21,22]. They form both homo and heterotrimeric receptors and contain two hydrophobic transmembrane domains spanning the plasma membrane. They have an intracellular N and C terminus and a large extracellular loop containing ~280 amino acids and 10 conserved cysteine residues that can form disulphide bridges to stabilize the protein structure. ATP binds to this extracellular region and activation of the channel can cause cells to become permeable to various small ions such as Na^+ and Ca^{2+} [21,22].

Purinergic receptors are present on a variety of malignant cells [23] and have displayed both pro-tumour and anti-tumour effects dependent on the cancer type and receptor expressed. Furthermore, studies have identified ATP to be at a high concentration in the tumour microenvironment yet low in surrounding healthy tissue [24]. This may be particularly the case in the bone tumour microenvironment where mechanical loading can stimulate ATP release from osteoblasts [25,26]. The aim of this review is to examine the evidence for P2 receptor expression in PBC, the downstream signalling pathways associated with P2 receptor activation and the functional consequences. The role of P2 receptors in PBC is summarised in this review, identifying the most promising avenues such as P2RX3 targeting for primary bone cancer induced pain (PBCP) and targeting P2RX4/P2RX7 on the primary tumour as a therapeutic option.

2. Methods

2.1 Search Strategy

This systematic review follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [27]. Articles published prior to December 10th, 2021 were searched in MEDLINE via OVID, PubMed, and the Web of Science core collection. Three searches were performed, one for each of the key topics within this systematic review; expression of P2 receptors in PBC, downstream signalling pathways associated with P2 activation and the functional consequences of P2 signalling. The extensive list of key search terms/alternative terms can be found in **Supplementary Tables 1,2,3**.

2.2 Inclusion and Exclusion Criteria

Eligibility criteria were determined beforehand. This review included only original articles written in English that had used (i) a PBC model and were (ii) investigating a P2 purinergic receptor. Reasons for excluding studies were as follows: (i) reviews, (ii) conference abstracts, (iii) publications in languages other than English, (iv) did not include a PBC model and (v) did not investigate a P2 purinergic receptor.

2.3 Selection of Articles to include in the Review

Articles retrieved from the databases based on the search strategy were screened for duplication, evaluated based on the titles and abstracts, and then finally full texts were read to determine whether or not the eligibility criteria were met. This was done independently by 2 of the authors, (LT and DCG), with the final list of articles to include agreed by all authors.

2.4 Risk of Bias Assessment and Quality Assessment

The risk of bias and quality assessment were adapted from a range of tools including Toxicological data Reliability Assessment Tool (ToxRTool) [28] and the Office of Health Assessment and Translation (OHAT) [29] risk of bias tool with guidance from the Cochrane Handbook [30]. The risk of bias further used SYRCLE's risk of bias tool [31] and the ARRIVE (Animal research: reporting *in vivo* experiments) guidelines [32] which were adapted and analysed for selection, performance, detection, attrition, reporting and other biases, characterising each study as low, moderate or high (full criteria **Supplementary Table 4**). The quality assessment used the Newcastle-Ottawa scale [33] and the National Institutes of Health (NIH) quality assessment tool [34] where a numerical score was generated with 0–3 low, 4–7 moderate and 8–10 high (full criteria **Supplementary Table 5**). These were performed by both LT and DCG independently, with a final outcome discussed and agreed upon for each individual publication.

2.5 Data Analysis

A narrative synthesis of included studies was performed to present the results for each key topic. Thematic grouping of studies was performed based on similar outcomes, interventions or comparable populations, using previously published systematic review guidance [35].

3. Results

3.1 Selection according to the PRISMA Guidelines

Across the three initial searches performed, 1191 articles were retrieved (Search 1 = 376, Search 2 = 299, Search 3 = 516, **Supplementary Tables 1,2,3**). These articles were

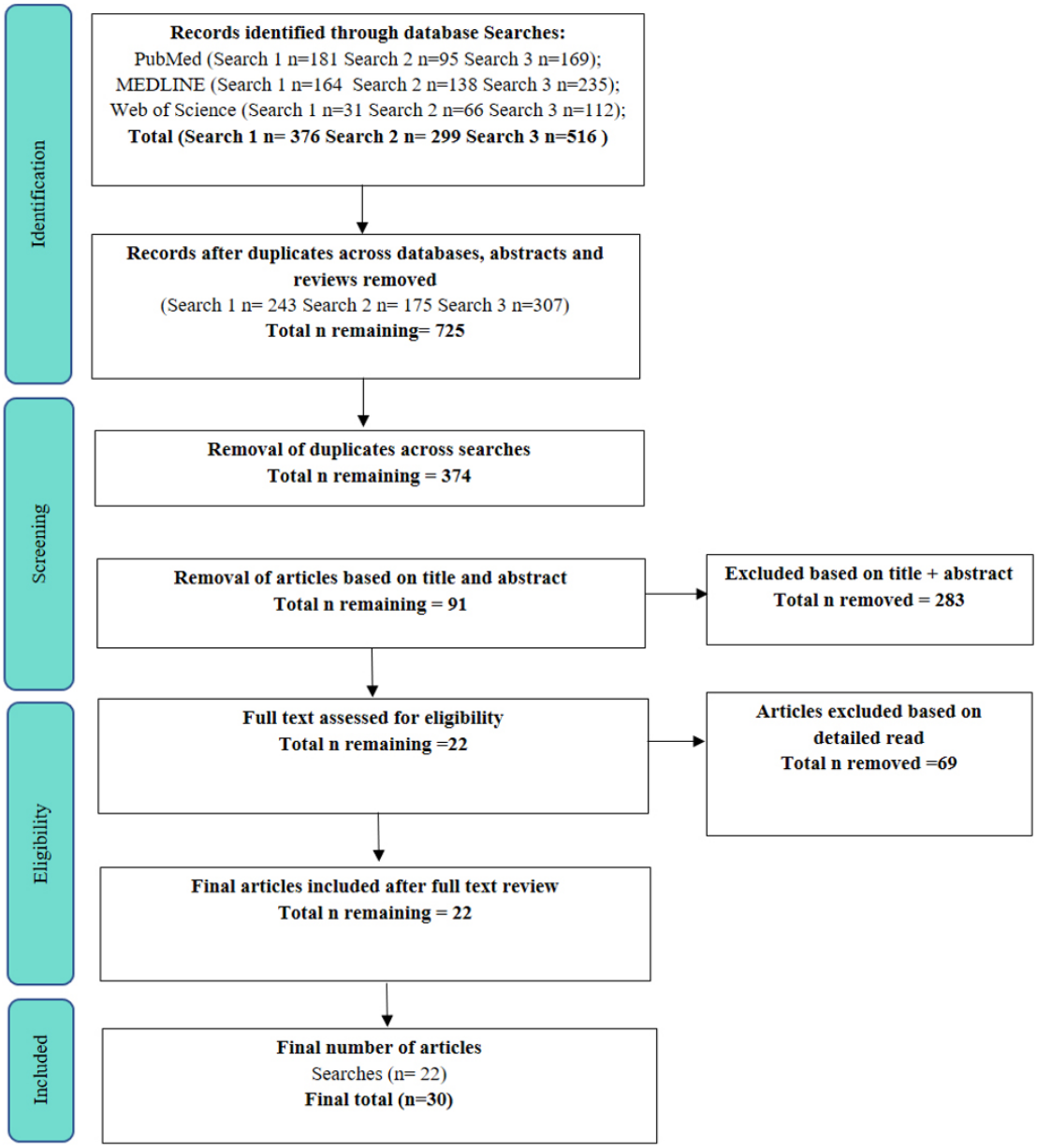

Fig. 1. PRISMA flow diagram including searching, deduplication, abstract screen, full text reads, excluded articles and final publication N number.

manually deduplicated across search databases and across multiple searches, screened for relevancy based on their title and abstracts, and assessed for inclusion and exclusion criteria upon reading the full-length article. Ultimately, 22 papers were included for appraisal. Supplementary manual snowballing [36] and hand-searching [37] were used to find 8 additional studies. In total, 30 studies are included within this systematic review, Fig. 1 summarises the selection process.

3.2 Risk of Bias and Quality Assessment

For the risk of bias *in vitro* and *in vivo*, selection, performance, detection, attrition, reporting and other bias were assessed for being low, moderate or high for each article. The criteria used to determine the extent of the bias is shown in **Supplementary Table 4**. For selection and performance bias for *in vitro* studies, all 26 studies were low [38–63], attrition bias was predominantly moderate, with some low [44–46] and the most poorly performing was reporting bias with 9 studies scoring high [38,39,43,54–57,59,63]. For *in vivo* studies, performance bias was the lowest [62–67], attrition and reporting bias were low and moderate apart from one study with high bias for reporting [65]. The most poorly performing areas were selection bias with three scoring high [65–67] and detection bias which again had three studies scoring high [64–66]. A full summary of the risk of bias for each study is shown in Table 1 (Ref. [38–67]).

Table 1. Risk of bias for the studies reviewed in this article.

	Bias Domain					
In vitro	Selection bias	Performance bias	Detection Bias	Attrition Bias	Reporting Bias	Other bias
Kumagai *et al*., 1991 [38]	Low	Low	Low	Low	High	Moderate
Reimer & Dixon., 1992 [39]	Low	Low	Low	Low	High	Low
Schofl *et al*., 1992 [40]	Low	Low	Low	Low	Moderate	Low
Yu & Ferrier., 1993 [41]	Low	Low	Low	Low	Moderate	Low
Gallinaro *et al*., 1995 [42]	Low	Low	Low	Moderate	Moderate	Low
Kaplan *et al*., 1995 [43]	Low	Low	Low	Low	High	Low
Bowler *et al*., 1995 [44]	Low	Low	Moderate	High	Low	Low
Urano *et al*., 1997 [45]	Low	Low	Low	High	Low	Moderate
Maier *et al*., 1997 [46]	Low	Low	Low	High	Moderate	Moderate
Luo *et al*., 1997 [47]	Low	Low	Low	Low	Low	Low
Jorgensen *et al*., 1997 [48]	Low	Low	Moderate	Moderate	Moderate	Low
Bowler *et al*., 1998 [49]	Low	Low	Moderate	Moderate	Moderate	Moderate
Bowler *et al*., 1999 [50]	Low	Low	Moderate	Low	Low	Low
Nakamura *et al*., 2000 [51]	Low	Low	Moderate	Moderate	Moderate	Low
Buckley *et al*., 2001 [52]	Low	Low	Moderate	Moderate	Moderate	Low
Gartland *et al*., 2001 [53]	Low	Low	Moderate	Low	Moderate	Low
Katz *et al*., 2006 [54]	Low	Low	Moderate	Low	High	Low
Hughes *et al*., 2007 [55]	Low	Low	Low	Low	High	Low
D'Andrea *et al*., 2008 [56]	Low	Low	Low	Low	High	Low
Alqallaf *et al*., 2009 [57]	Low	Low	Moderate	Moderate	High	Low
Liu & Chen., 2010 [58]	Low	Low	Low	Low	Moderate	Low
Giuliani *et al*., 2014 [59]	Low	Low	Moderate	Moderate	High	Low
Qi *et al*., 2016 [60]	Low	Low	Moderate	Low	Medium	Low
Wang *et al*., 2019 [61]	Low	Low	Moderate	Moderate	Low	Low
Zhang *et al*., 2019 [62]	Low	Low	Moderate	Moderate	Moderate	Low
Tattersall *et al*., 2021 [63]	Low	Low	Moderate	Low	High	Low
In vivo						
Gonzales-Rodriguez *et al*., 2009 [64]	High	Low	High	Low	Low	Low
Hansen *et al*., 2011 [65]	High	Low	High	Low	High	Low
Guedon *et al*., 2016 [66]	High	Low	Low	Low	Moderate	Low
Zhang *et al*., 2019 [62]	Moderate	Low	High	Moderate	Moderate	Low
He *et al*., 2020 [67]	Low	Moderate	Low	Low	Moderate	Low
Tattersall *et al*., 2021 [63]	Moderate	Low	Low	Moderate	Moderate	Low

Risk of bias determined for each bias domain-low risk of bias, moderate risk of bias or high risk of bias.

The quality assessment was performed for both *in vitro* and *in vivo* studies, all 30 articles included were peer reviewed. The criteria used to determine the quality of each article is shown in **Supplementary Table 5**. Although the studies discussed the background and how results were obtained clearly, only 4 explicitly stated an aim [43,46,57,59]. The majority of studies (22 out of 30) clearly stated the cell source, e.g., a previous lab group or commercially bought, and 8 studies did not [40,41,44,45,48,53,61,62]. Biological and technical repeats were present in 14 *in vitro* studies [38–41,43,47,50,53–55,58,60,63] with 9 including only information regarding biological [42,44,48,51,52,55,59,61,62] and three providing neither [44–46]. All *in vivo* studies in this review stated mice N numbers [62–67].

The quality of the data in each study was assessed for exclusions. For *in vitro* studies, 16 included 'data not shown' [38,39,43,46,47,49,51–59,63] and of the 6 *in vivo* studies three included 'data not shown' [63,65,66]. For the 26 *in vitro* studies 14 had representative data [38–43,54–57,59,60,62,63] and 12 did not specify [44–53,58,61]. For *in vivo* studies 4 had representative data [62,63,65,67] and two did not specify [64,66]. The studies were assessed for the statistical analysis performed where 16 *in vitro* studies stated statistical *p* values [39,41,43,47,49–51,53,54,57–63] and 10 did not [38,40,41,44–46,48,52,55,56]. All 6 *in vivo* studies stated statistical *p* values [62–67]. Finally, the inclusion of limitations and future studies in the discussion was assessed of which 5 *in vitro* studies [40,41,43,48,52] and one *in vivo* study [67] had clear limitations. Future studies were clearly stated in 5 *in vitro* studies [43,44,54,58,63] and two *in vivo* studies [63,67]. Of the 26 *in vitro studies* 19 were determined to be of moderate quality [38–42,44–

Table 2. Quality assessment scores for the studies reviewed in this article.

Study	Quality assessment criteria										
In vitro	1	2	3	4	5	6	7	8	9	10	Total
Kumagai *et al*., 1991 [38]	Yes	Yes	No	Yes	Yes	No	No	No	Yes	Yes	6
Reimer & Dixon., 1992 [39]	Yes	No	No	Yes	Yes	No	Yes	No	Yes	Yes	6
Schofl *et al*., 1992 [40]	Yes	No	Yes	Yes	Yes	Yes	No	Yes	No	Yes	7
Yu & Ferrier., 1993 [41]	Yes	No	No	Yes	Yes	Yes	No	Yes	Yes	Yes	7
Gallinaro *et al*., 1995 [42]	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	7
Kaplan *et al*., 1995 [43]	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	9
Bowler *et al*., 1995 [44]	Yes	No	Yes	Yes	No	Yes	No	Yes	No	Yes	6
Urano *et al*., 1997 [45]	Yes	No	Yes	Yes	No	Yes	No	No	Yes	Yes	6
Maier *et al*., 1997 [46]	Yes	Yes	Yes	Yes	No	Yes	No	No	No	Yes	6
Luo *et al*., 1997 [47]	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	7
Jorgensen *et al*., 1997 [48]	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes	7
Bowler *et al*., 1998 [49]	Yes	No	Yes	Yes	No	Yes	Yes	No	No	Yes	6
Bowler *et al*., 1999 [50]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	8
Nakamura *et al*., 2000 [51]	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	7
Buckley *et al*., 2001 [52]	Yes	Yes	No	Yes	No	Yes	No	Yes	Yes	Yes	7
Gartland *et al*., 2001 [53]	Yes	No	Yes	Yes	Yes	Yes	Yes	No	No	Yes	7
Katz *et al*., 2006 [54]	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	8
Hughes *et al*., 2007 [55]	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes	7
D'Andrea *et al*., 2008 [56]	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes	7
Alqallaf *et al*., 2009 [57]	Yes	Yes	Yes	Yes	No	No	Yes	No	No	Yes	6
Liu & Chen., 2010 [58]	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9
Giuliani *et al*., 2014 [59]	Yes	Yes	Yes	Yes	No	No	Yes	No	No	Yes	6
Qi *et al*., 2016 [60]	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	8
Wang *et al*., 2019 [61]	Yes	No	Yes	Yes	No	Yes	Yes	No	No	Yes	6
Zhang *et al*., 2019 [62]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	9
Tattersall *et al*., 2021 [63]	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	9
In vivo											
Gonzales-Rodriguez *et al*., 2009 [64]	Yes	Yes	No	Yes	No	Yes	Yes	No	No	Yes	7
Hansen *et al*., 2011 [65]	Yes	Yes	No	Yes	No	No	Yes	Yes	No	Yes	6
Guedon *et al*., 2016 [66]	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	8
Zhang *et al*., 2019 [62]	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	7
He *et al*., 2020 [67]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	10
Tattersall *et al*., 2021 [63]	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	9

Quality Assessment: 0–3 = Low quality, 4–7 = Moderate quality, 8+ = High quality.

49,51–53,55–57,59,61] and 7 were determined to be high quality [43,50,54,58,60,62,63]. For *in vivo* studies three were determined to be of moderate quality [62,64,65] and three were determined to be high quality [63,66,67]. Overall, as no studies were scored low, all studies were determined to be of acceptable quality and included in the review. A summary of the quality assessment for each study can be found in Table 2 (Ref. [38–67]).

3.3 PBC Models and P2 Receptors included in this Review

In this review 26 studies perform *in vitro* investigations using PBC cell lines. These cell lines include UMR106 [38,39,41–43,47,48,50,52], ROS17/2.8 [40,43,48,54–56], SaOS-2 [44,50,52,56,57,62], U2OS [60,62], MG-63 [46,51,57,62], TE85 [44,53,59,63], MNNG-HOS [62,63], HOS unspecified [58], OHS-4 [46], NY [45] and HuO3N1 [45] with two studies investigating a chondrosarcoma cell line- SW1353 [61,62]. A total of 6 studies performed *in vivo* investigations of OS-induced mouse models. Two studies use a xenograft model achieved through inoculating BALB/c nude mice with MNNG-HOS [62,63] and one also used a tail vein injection of MNNG-HOS cells as a lung colonisation model [63]. The other four studies use syngeneic mouse models achieved through inoculating 6-9-week-old C3H/HeJ mice with NCTC 2472 cells [64–67]. Finally, 5 studies utilised patient tissue samples. Two of those 5 studies included stage IV OS samples [59,62]. One had 52 samples (39 male and 15 female patients aged 5 to 61) [59] and one had 10 samples (7 male and three female aged 10–50) [62]. The other three studies collected human tissue from GCTB patients [44,49,50], with an individual patient being used for each study. Out of these three stud-

ies, patient information was disclosed in only one study, where tissue was derived from a 71-year-old male [44]. Of the 30 studies reviewed 6 did not specify which P2 receptor was investigated [38–42,55], 12 articles investigated P2Y receptors *in vitro*; P2RY1 [46,50,52,54,56], P2RY2 [43,44,46–50,52,54,56,58], P2RY4 [46,58], P2RY5 [58], P2RY6 [46], P2RY9 [58], P2RY11 [58,61], 9 articles investigated P2X receptors *in vitro*; P2RX4 [51,57,58], P2RX5, [51], P2RX6 [45,51], P2RX7, [51,54,57–60,62,63]. One article investigated both P2Y and P2X receptors (P2RX4, P2RX7, P2RY2, P2RY4, P2RY5, P2RY9, P2RY11) [58]. All 6 *in vivo* studies investigated P2X receptors; P2RX3 [64,66,67] P2RX7A [62,65] P2RX7B [63] and P2RX7K [65].

3.4 Non-PBC Comparators included in this Review

In this review 26 studies performed *in vitro* experiments, 10 studies had multiple non-PBC comparators [44–46,48,50,53,55,57,61], 10 had a single non-PBC comparator [39,40,43,47,49,55,58,59,62,63], and in 7 studies non-PBC comparators were absent [38,41,42,51,52,54,60]. The most common non-PBC comparators were human bone derived osteoblast cells which were included in 5 studies [40,46,47,50,53], with primary osteoclasts [47], human osteoblast like HOBIT cells [56], MC3T3-E1 murine osteoblasts [43], and human bone marrow mesenchymal stem cells [62] each used in one study. Other sarcoma cell lines were used with both rhabdomyosarcoma (A204, A673, Hs729T, RD) and liposarcoma (SW872) included in one study [45], with human chondrocytes used in the single chondrosarcoma study [61]. Other non-osteoblast cells were used in 12 studies [39,44,46,48,49,53,55,57–59,61,62]. Of the 6 *in vivo* studies, 4 had sham non tumour bearing mice comparators [64–67].

3.5 Techniques used to Assess Experimental Outcomes

A variety of different experiments were used to confirm P2 receptor expression, function and signalling. To investigate P2 expression at a molecular level RT-PCR was used in 10 studies [44–47,50,51,54,57,61,62], qPCR in three studies [59,62,63], northern blotting in three studies [45,46,48], Southern blotting [44] and *in situ* hybridisation in one [49]. To determine P2 protein expression immunohistochemistry (IHC) was used in 5 studies [59,62,65–67], immunocytochemistry in three [53,57,60], western blotting in 4 [57,61,62,67], and flow cytometry [59] and patch clamping [67] in one study respectively. To determine receptor functionality 18 studies assessed Ca^{2+} response to stimulation with ATP, BzATP, ADP, 2-MeSATP or UTP across a range of concentrations [38–44,47,48,50–52,54–56,58,59,63]. A further 5 studies also assessed P2RX7-mediated cationic or anionic dye uptake, three by ethidium bromide [53,59,63], one using YO-PRO [56] and one using lucifer yellow/calcein [60]. For investigations into P2 signalling pathways 9 different studies in total assessed P2 signalling, of those, 5 studies identified different interventions that could affect calcium signalling using dye-loaded cells [43,50,52,56,58]. Five studies identify specific downstream signalling pathways [50,52,59,62,63], with two studies using luciferase reporter gene assays for gene induction [44,52] two studies use enzyme linked immunosorbent assay (ELISA) [59,62], a northern blot [50], western blot [62] and RNA-seq [63] were also used once. Finally, one *in vivo* study used western blotting and ELISA to identify P2 receptor trafficking downstream of other signalling [67].

To investigate functional consequences of P2 signalling *in vitro*, 6 studies perform proliferation assays [51,58–60,62,63], with three assessing cell death [53,58,60], two further included migration and invasion [62,63] and one cell adhesion [63]. Further to this, one included IHC on PBC patient tissue where the level of Ki-67 staining as a marker of cell growth was assessed in samples expressing different P2 receptors [59]. Out of 26 *in vitro* studies 14 used an antagonist, with suramin being the most commonly used in 6 studies [48,51,54–56,58], KN62 and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) in three [51,57,62], and A740003 used in three [59,62,63], with all other antagonists used once and include other nucleotides for cross reactivity [41], BBG [56], oATP [57], reactive blue 2 [51], NF157 [61] and BBP [58]. A total of 6 studies had *in vivo* experiments with 4 of them investigating PBCP [64–67] and two investigating PBC treatment [62,63]. The 4 that measured pain response as the experimental outcome used mechanical allodynia (using von Frey monofilaments) in three studies [65–67], thermal hyperalgesia in three studies [64,65,67], and limb use and weight bearing assessment in two studies [65,66]. One study included spontaneous nocifensive behaviours (such as flinching) and tail flick assays [66] and one study included formalin-induced pain and spared nerve injury [65]. Aside from pain behaviour techniques three studies included IHC [65–67], and one study used micro-CT scanning, histology and an immunoblot [65] in order to determine the different functional effects. For the two *in vivo* studies examining PBC treatment, the techniques used to determine the functional effects were IHC, micro-CT scanning and histology [62,63]. All 6 *in vivo* studies used antagonists, A740003 for PBC treatments [62,63] and A317491 [64,67], Anti-P2RX3 antibody [66] and A438079 [65] for PBCP.

3.6 Narrative Synthesis

3.6.1 Expression of P2 Receptors in PBC *in vitro* Murine Models

Initial studies into P2 receptors in OS did not specify which receptors were expressed but that it was possible to detect calcium elevation using various P2 receptor agonists. This was the case using the rat OS cell line UMR106 where P2 receptor expression was reported due to a dose

dependent Ca^{2+} elevation in response to purine nucleotides ATP and ADP, but also the pyrimidine nucleotide UTP [38]. Studies that followed then established subtypes of purinergic receptors responsible for this Ca^{2+} signal where they were shown to contain a subtype responsive to ATP, 2-MeSATP and ADP (P2Y1R) and another responsive to ATP and UTP (P2RY2) [39,41–43]. Further P2RY2 activity was shown [47] but another study ultimately established P2RY1 as the predominant subtype in UMR106 cells based on potency of the agonist applied [52].

P2 receptor expression within another rat OS cell line (ROS17/2.8) was again demonstrated by showing Ca^{2+} activation caused by endogenous P2Y subtypes with as little as 10 μM UTP, ATP and ADP [54]. However, it was also found that ROS17/2.8 cells were insensitive to 200 μM ATP with no Ca^{2+} response observed [40]. This finding is supported as 100 μM UTP, ATP and ADP [56] and 200 μM UTP or ATP [55] failed to produce a response. In only one study both UMR106 and ROS17/2.8 cells where used. The study showed that UMR106 but not ROS17/2.8 cells responded by producing Ca^{2+} responses when treated with ATP or UTP, although the data for ROS17/2.8 was not presented [48]. This study also demonstrated P2RY2 was expressed in UMR106 cells but not ROS17/2.8 cells using a northern blot.

3.6.2 Expression of P2 Receptors in PBC *in vitro* Human Models

P2 expression has been found in human OS cell lines, one study provides general evidence for P2 activity in SaOS-2 cells, without assigning activity to either P2Y or P2X subtypes [40]. Three further studies focus on P2Y expression in SaOS-2 cells with each confirming P2Y receptor activity. P2RY1 was implicated as the main P2Y subtype [56] although this was not characterised at a molecular level and was based on Ca^{2+} response due to its sensitivity to ATP and insensitivity to UTP. It was subsequently shown that P2RY2 at the mRNA level is present in SaOS-2 and TE85 cells using RT-PCR [44]. Overall, a final study regarding P2RY1 and P2RY2 expression in SaOS-2 cells confirmed P2RY1 expression was at a greater level in SaOS-2, compared to P2RY2 at a molecular level, again using RT-PCR [50]. Regarding other P2Y receptors in SaOS-2 and TE85 cells no studies provide further results, however, MG-63 and OHS-4 cells were reported as having P2RY1, P2RY2, P2RY4, P2RY6 expression by RT-PCR, but the data wasn't shown [46].

Eight studies have investigated P2X signalling across a wider range of human PBC cell lines with a predominant focus on P2RX7. Early work demonstrated both expression and function of this receptor in MG-63 cells by RT-PCR, qRT-PCR, western blot, immunofluorescence, pore formation and Ca^{2+} measurement. P2RX7 was found to be present at mRNA and protein level and be functional in these cells [51,57,62]. This is similarly the case for SaOS-2 cells where P2RX7 has been shown again by RT-PCR, qRT-PCR, western blot, immunofluorescence, pore formation and Ca^{2+} measurement to have expression at mRNA and protein levels, as well as being functional [53,57,62]. In U2OS cells in one study P2RX7 gene expression data wasn't shown, however P2RX7 protein expression by immunofluorescence and pore formation was shown [60], and was subsequently confirmed in a further study for mRNA expression by qRT-PCR and protein by western blotting [62]. Further studies have investigated P2RX7 in TE85 OS cells showing that P2RX7 mRNA is low/absent in this cell line [53,59,63] using RT-PCR and qRT-PCR. The receptor was undetectable at the protein/functional level in all 3 studies when assessed using immunocytochemistry, Ca^{2+} response and pore formation [53,59,63]. Typical P2RX7 Ca^{2+} responses were shown with transfection of P2RX7A or P2RX7B [59,63] and pore formation was only demonstrated when TE85 cells were transfected with both isoforms [59], in addition flow cytometry was used to determine which isoform had the most protein expression in transfected cells [59]. Despite the parental TE85 cell line not having mRNA or protein expression of P2RX7, one study has shown that its derivative cell line MNNG-HOS did have P2RX7 mRNA and protein expression using a qRT-PCR and western blot with its function also demonstrated using Ca^{2+} and pore formation assays [62]. In contrast to this, another study did not find P2RX7 expression in MNNG-HOS cells using RT-PCR, qPCR, Ca^{2+} or pore formation techniques [63]. Regarding other P2X receptors; P2RX1, P2RX2 and P2RX3 were shown to be absent and P2RX4, P2RX5 and P2RX6 present at the mRNA level in MG-63 cells using RT-PCR [51]. A second study also showed that P2RX2 was absent and P2RX4 was present at the mRNA and protein level using RT-PCR and western blotting in MG-63 cells and SaOS-2 cells [57]. P2RX6 has also been shown to be expressed in a further two OS cell lines NY and HuO3N1 using RT-PCR and northern blotting [45]. Only one study analysed all of the P2Y and P2X receptors in human OS cells, using HOS cells where P2RX4, P2RX7, P2RY2, P2RY4, P2RY5, P2RY9 and P2RY11 mRNA was detected by RT-PCR [58], however, the specific cell type (MNNG-HOS, TE85-HOS or KHOS) was not detailed.

Finally, two studies show P2 expression in the SW1353 chondrosarcoma cell line, this cell line expresses P2RX7 at the mRNA and protein levels shown by qRT-PCR and western blotting [62], although functionality of this receptor in these cells has not yet been explored. This was the same for P2RY11 in SW1353 cells which had mRNA expression using RT-PCR and protein expression using western blotting but no further functional studies [61]. There is no expression data regarding any P2 receptors in either Ewing's sarcoma or chordoma. A full summary of P2 expression *in vitro* for all studies is shown in Table 3 (Ref. [38–63]).

3.6.3 Expression of P2 Receptors in PBC *in vivo*

Four studies investigating PBCP targeted P2RX3 and P2RX7 in NCTC2472 mouse models through receptor inhibition. One study inhibited P2RX3 with anti-P2RX3 antibody using IP injection 7 days after cell inoculation and every 5 days thereafter. Although this study showed P2RX3 was detected (using IHC) in the L2 dorsal root ganglia that innervate the tumour-bearing femur, there was no focus on demonstrating expression on the primary bone tumour [66]. This was a similar case in another study where P2RX3 was targeted using A317498 administered as a peritumoral injection 30 minutes before observation of mouse pain behaviours, although again no techniques were used to explicitly show expression of P2RX3 in the primary tumour [64]. A final study investigated P2RX3 trafficking in PBCP, P2RX3 was targeted by A317498 administered a week after cell inoculation by intrathecal injection daily, assessment of P2RX3 was performed on L3-L5 Dorsal root ganglion (DRG) neurons. P2RX3 protein was confirmed in the neurons using western blotting and immunofluorescence with its function shown using patch clamp recordings [67] but again had no analysis of the primary tumour was performed. These studies show that targeting P2RX3 for PBCP is through modification of the bone tumour microenvironment/the pain response pathways and not the tumour itself as this hasn't been assessed. Aside from P2RX3 targeting for PBCP, P2RX7 and its role in pain has also been examined in one study. OS bearing P2RX7 knock out (KO) mice were susceptible to PBCP and had an earlier onset of pain-related behaviours compared with cancer-bearing wild-type mice. Treatment with A438079 (administered subcutaneously on various different days depending on which pain behaviour was been assessed) did not alleviate pain related behaviours in models of PBCP. When analysing the mice, the KO mice were identified as having P2RX7 mRNA expression in spinal cord tissue but not in osteoclasts; western and immunoblotting were then used to confirm protein expression although this was not determined for different isoforms, and the study did not focus on P2RX7 expression on the primary tumour itself [65]. Only two studies target the P2RX7 (full length and P2RX7B) expressed on the PBC tumour as a potential therapeutic option by treating MNNG-HOS OS cells with the P2RX7 agonist BzATP [62] and antagonist A740003 [62,63]. P2RX7 expression and function was confirmed on the cells (detailed in 3.6.2 *in vitro* section) before the intra-tibia, paratibial and tail vein injection [62,63]. The effect of targeting any P2 receptors either as a direct tumour target or to control PBCP in Ewing's sarcoma, chondrosarcoma or chordoma using *in vivo* models has not been demonstrated. A full summary of P2 expression *in vivo* for all studies is shown in Table 4 (Ref. [62–67]).

3.6.4 Expression of P2 Receptors in Clinical Samples of PBC

Three studies identify P2RY1 and P2RY2 in GCTB. The first identified P2RY2 mRNA using RT-PCR and Southern blotting in tissue from a single patient [44]. The second identified P2RY2 transcript localisation in the osteoclast population of a tumour surgically removed from a patient using *in situ* hybridisation, but although the transcript was detected it was found absent on the surface of the primary cells as a functioning receptor [49]. In the final study, along with P2RY2, P2RY1 expression was shown in a GCTB tumour again by RT-PCR but did not include further functional experiments [50]. No other P2Y receptors have been analysed in other PBC patient samples.

Two studies show P2RX7 expression in samples derived from OS patients [59,62]. The first screened a panel of 54 stage IV OS samples for expression of either P2RX7A or P2RX7B splice isoforms using IHC. These differ due to the lack of a C-terminal tail on the P2RX7B, meaning separate antibodies can be used to target the N-terminal region detecting both P2RX7A and P2RX7B, whereas antibodies directed to the C-terminal region will only be present when the full length receptor P2RX7A is present, and therefore this can distinguish between the two. These isoforms were expressed in 81% of tumour samples [59] whilst a subsequent study also using stage IV OS samples (n = 10) found P2RX7 expression to be highly expressed when compared to normal bone samples from patients having a hip replacement [62]. None of the studies identified have examined any other P2X receptors in OS or other PBCs. A full summery of P2 expression in clinical samples for all studies is shown in Table 5 (Ref. [44,49,50,59,62]).

3.6.5 P2 Receptor Associated Signalling Pathways in PBC

3.6.5.1 Modulation of P2-mediated Ca^{2+} Signalling in PBC. Five studies describe the ability of other non-nucleotide molecules to modulate Ca^{2+} signalling associated with P2 receptors, each assessing changes in Ca^{2+} as a primary outcome [43,50,52,56,58]. Three of those studies address potential synergy between extracellular nucleotides and the systemic parathyroid hormone (PTH) with differences in their findings. Two studies describe a synergistic effect whereby PTH can potentiate nucleotide induced Ca^{2+} release from intracellular stores in UMR106 cells [43,52]. The first demonstrates this synergy with ATP and UTP indicative of P2RY2 receptor signalling [43] whereas the second focusses on the synergy between the P2RY1 agonist ADP and PTH [52]. However both provide a differing report whereby in the first study, 100 μM PTH alone can induce Ca^{2+} elevations [41] but in the second 0.1–500 ng/mL PTH was shown that it could not [52]. In contrast to both

Table 3. Expression of P2 receptors in PBC *in vitro* in murine and human models.

P2 expression in cell lines							
Study	PBC model	mRNA detection	Protein detection	Functional detection	Non-PBC comparator	Antagonist	Main findings
Kumagai *et al*., 1991 [38]	UMR106	None	None	Fluorescent Ca^{2+} assay	None	No	P2 receptors present on UMR106 which are sensitive to ATP, ADP and UTP.
Reimer & Dixon., 1992 [39]	UMR106	None	None	Fluorescent Ca^{2+} assay	Platelets	No	P2 receptors present which are sensitive to ATP, 2-MeSATP, ADP and UTP.
Schofl *et al*., 1992 [40]	ROS17/2.8, SaOS-2	None	None	Fluorescent Ca^{2+} assay	Primary osteoblasts	No	P2 receptors present on SaOS-2 cells which are sensitive to ATP. No response with ROS17/2.8.
Yu & Ferrier., 1993 [41]	UMR106	None	None	Fluorescent Ca^{2+} assay	None	Yes-other nucleotides	P2 receptors present on cells which respond to ATP, 2-MesATP and UTP.
Gallinaro *et al*., 1995 [42]	UMR106	None	None	Fluorescent Ca^{2+} assay	None	No	P2 receptors present which respond to ATP, 2-MeSATP, ADP and UTP.
Kaplan *et al*., 1995 [43]	UMR106	None	None	Fluorescent Ca^{2+} assay	MC3T3-El mouse osteoblast cell line	No	P2RY2 receptors present which respond to ATP and UTP.
Bowler *et al*., 1995 [44]	SaOS-2, TE85	RT-PCR* Southern blotting*	None	None	Macrophage, primary human bone derived cells	No	SaOS-2 and TE85 express P2RY2 at the mRNA level.
Urano *et al*., 1997 [45]	NY and HuO3N1	Northern Blot * RT-PCR *	None	None	Rhabdomyosarcoma: A204, A673, Hs729T, RD & liposarcoma: SW872	No	P2RX6R transcript present in NY and HuO3N1.
Maier *et al*., 1997 [46]	MG-63	Northern Blot * RT-PCR $	None	None	SK-N-SH, U138 and H4 Y79, U87, CI-215 118-INI-Brain-derived cell lines. Primary human bone sample	No	P2RY1, P2RY2, P2RY4, P2RY6 expressed in MG-63 and OHS-4 cells.
Luo *et al*., 1997 [47]	UMR106	None	None	Fluorescent Ca^{2+} assay	Primary osteoclasts	No	Cells respond to ATP and UTP suggesting P2RY2 expression.
Jorgensen *et al*., 1997 [48]	UMR106 ROS17/2.8	Northern Blot*	None	Fluorescent Ca^{2+} assay	Hamster tracheal epithelia cells, mouse macrophage like cell line J774	Yes-suramin	P2RY2 expressed at the mRNA level in UMR106 but not ROS17/2.8.
Bowler *et al*., 1999 [50]	SaOS-2 UMR106	RT-PCR*	None	Fluorescent Ca^{2+} assay	2 populations of Primary human bone derived cells	No	P2RY1 has higher expression than P2RY2 in SaOS-2 cells.
Nakamura *et al*., 2000 [51]	MG-63	RT-PCR̂	None	Fluorescent Ca^{2+} assay	None	Yes-suramin, reactive blue 2, PPADS	MG-63 cells express P2RX4, P2RX5, P2RX6 and P2RX7 but not P2RX1, P2RX2, or P2RX3.
Buckley *et al*., 2001 [52]	UMR106	None	None	Fluorescent Ca^{2+} assay	None	No	P2Y1R expressed in UMR106 cells.

Table 3. Continued.

P2 expression in cell lines							
Study	PBC model	mRNA detection	Protein detection	Functional detection	Non-PBC comparator	Antagonist	Main findings
Gartland *et al*., 2001 [53]	SaOS-2, TE85	RT-PCR*	IHC	Ethidium bromide uptake	Primary human bone derived cells, THP-1 cells	Yes-PPADS	SaOS-2 cells express PR2X7 at the mRNA and protein levels. TE85 expresses P2RX7 mRNA but not protein.
Katz *et al*., 2006 [54]	ROS17/2.8	None	None	Fluorescent Ca^{2+} assay	None	Yes-suramin	P2RY1 and P2RY2 receptor are present sensitive to ATP, ADP and UTP.
Hughes *et al*., 2007 [55]	ROS17/2.8	None	None	Fluorescent Ca^{2+} assay	COS7 (Kidney fibroblast like) HEK-239	Yes-suramin	ROS17/2.8 not responsive to UTP or ATP.
D'Andrea *et al*., 2008 [56]	ROS17/2.8, SaOS-2	None	None	Fluorescent Ca^{2+} assay	HOBIT Human osteoblast like	Yes-suramin	SaOS-2 cells are sensitive to ATP, ADP and UTP. ROS17/2.8 are not.
Alqallaf *et al*., 2009 [57]	SaOS-2, MG-63	RT-PCR*	Western Blot immunofluorescence	YO-PRO1 uptake	BON1 (Human Pancreatic), HEK-293+P2RX7	Yes-BBG, KN-62, oATP and PPADS	Both cell lines express P2RX4 and P2RX7 at the mRNA and protein levels, P2RX2 was absent in both cell lines.
Liu & Chen., 2010 [58]	HOS	RT-PCR #	None	Fluorescent Ca^{2+} assay	PC12- Rat pheochromocytoma cell line	Yes-suramin, BBP	HOS cells express P2RX4, P2RX7, P2RY2, P2RY4, P2RY5, P2RY9 and P2RY11 at the mRNA level.
Giuliani *et al*., 2014 [59]	TE85	RT-PCR*	IHC Flow cytometry	Ethidium bromide uptake	HEK293+P2RX7	Yes-A740003	P2RX7 not endogenously expressed in TE85 cells but were transfected with P2RX7A and P2RX7B splice isoforms.
Qi *et al*., 2016 [60]	U2OS	None	Immunofluorescence	Lucifer yellow and calcein uptake	None	Yes-KN62	U2OS cells express P2RX7 at the protein level.
Wang *et al*., 2019 [61]	SW1353	RT-PCR*	Western Blot	None	Huh-7 human hepatocellular carcinoma, Human chondrocytes	Yes- NF157	SW1353 cells express P2RY11 at the mRNA and protein level.
Zhang *et al*., 2019 [62]	SaOS-2, U2OS, MNNG-HOS, MG-63, SW1353	qRT-PCR*	Western Blot	None	Human bone marrow mesenchymal stem cells	Yes-A740003	P2RX7 is expressed by all cell lines at the mRNA and protein levels. Expression is highest in MNNG-HOS and lowest in SaOS-2.
Tattersall *et al*., 2021 [63]	TE85, MNNG-HOS	RT-PCR, qRTPCR*	None	Fluorescent Ca^{2+} assay, ethidium bromide uptake	HEK-293+P2RX7A	Yes-A740003	P2RX7 not endogenously expressed in TE85 and MNNG-HOS cells but were transfected with P2RX7B splice isoform.

* Specific receptor only; ^ All P2X; $ All P2Y; # All P2X and P2.

Table 4. Expression of P2 receptors in PBC *in vivo*.

		P2 expression *in vivo*					
Study	PBC model	mRNA detection	Protein detection	Functional detection	Non-PBC comparator	Antagonist	Main findings
Gonzales-Rodriguez *et al*., 2009 [64]	Syngeneic mouse NCTC 2472	None	None	PBCP- thermal hyperalgesia.	Sham mice	Yes-A317491	P2RX3 expression not explicitly explored besides assessment of bone pain behaviour.
Hansen *et al*., 2011 [65]	Syngeneic mouse NCTC 2472	None	IHC	PBCP- thermal hyperalgesia, mechanical allodynia, spared nerve injury, formalin-induced pain, limb use and weight bearing. IHC, micro-CT, histology, immunoblot.	Sham mice	Yes-A438079	P2RX7A and P2RX7K identified in spinal cord tissue. A438079 did not reduce PBCP.
Guedon *et al*., 2016 [66]	Syngeneic mouse NCTC 2472	None	IHC	PBCP- mechanical allodynia limb use and weight bearing, tail flick assay, spontaneous nocifensive behaviours such as flinching. IHC.	Sham mice	Yes-Anti-P2RX3 antibody	P2RX3 expressed in the L2 dorsal root ganglia that innervate the tumour-bearing femur.
Zhang *et al*., 2019 [62]	Xenograft mouse MNNG-HOS	None	None	IHC, micro-CT and histology.	None	Yes-A740003	P2RX7 shown to be present and functional on the cells before injection, expression not explicitly explored *ex vivo*. However IHC, micro-CT and histology assessed P2RX7 effects on the tumour, bone and lung metastasis.
He *et al*., 2020 [67]	Syngeneic mouse NCTC 2472	None	Patch clamp, Immunofluorescence, western blot	PBCP- thermal hyperalgesia, mechanical allodynia. IHC.	Sham mice	Yes-A317491	P2RX3 protein was confirmed L3-L5 DRG neurons.
Tattersall *et al*., 2021 [63]	Xenograft mouse MNNG-HOS+P2RX7B Tail vein MNNG-HOS +P2RX7B	None	None	IHC, micro-CT and histology.	None	Yes-A740003	P2RX7B transfected into the cells before injection, expression not explicitly explored *ex vivo*. However IHC, micro-CT and histology assessed P2RX7B effects on the tumour, bone and lung metastasis.

Table 5. Expression of P2 receptors in PBC in clinical samples.

		P2 expression in patient tissue					
Study	PBC model	mRNA detection	Protein detection	Functional detection	Non-PBC comparator	Antagonist	Main findings
Bowler *et al.*, 1995 [44]	GCTB (n = 1)	RT-PCR *	None	None	Normal bone tissue	No	P2RY2 expressed in GCTB.
Bowler *et al.*, 1998 [49]	GCTB (n = 1)	*In situ* hybridization *	None	Ca^{2+} measurement	None	No	P2RY2 expressed in the osteoclast population of GCTB.
Bowler *et al.*, 1999 [50]	GCTB (n = 1)	RT-PCR*	None	None	Primary human bone derived cells	No	P2RY1 expressed in GTCB.
Giuliani *et al.*, 2014 [59]	OS Stage IV (n = 54)	None	IHC	None	None	Yes-A740003	P2RX7A and P2RX7B expressed in 81% of samples.
Zhang *et al.*, 2019 [62]	OS Stage IV (n = 10)	None	IHC	None	Normal bone tissue	Yes-A740003	P2RX7 is highly expressed in 10 OS samples when compared to 2 normal bone samples from patients having hip replacements.

* Specific receptor only; ^ All P2X; $ All P2Y; # All P2X and P2Y.

these studies, another study demonstrated that there was no synergistic Ca^{2+} elevation between PTH and nucleotides (ATP and UTP) in SaOS-2 cells; and that 20–500 ng/mL PTH alone did not produce a response [50].

One study investigated H_2O_2, a reactive oxygen species, for its potential role in regulating Ca^{2+} homeostasis [56]. Wild-type ROS17/2.8 cells lacking endogenous expression of P2RY2 display no response to extracellular nucleotides or H_2O_2. Stable transfection of P2RY2 in these cells results in sensitivity to extracellular nucleotides through which micromolar concentrations of H_2O_2 can potentiate Ca^{2+} release from thapsigargin-sensitive stores. This effect could not be replicated in P2RY1-predominant SaOS-2 cells even when stimulated with millimolar concentrations of H_2O_2. Thus, it was suggested a mechanism whereby oxidation of P2RY2 cysteine residues can sensitize the receptor to lower agonist concentrations [56].

The final study used an array of phthalates to show their ability to suppress ATP-mediated Ca^{2+} elevations. The most potent at doing so was butyl benzyl phthalate (BBP) which blocked Ca^{2+} influx associated with P2X activity [58]. A summary of studies showing modulation of P2-mediated Ca^{2+} signalling in PBC are listed in Table 6 (Ref. [43,50,52,56,58,59,62,63,67]).

3.6.5.2 Modulation of Gene Pathways in PBC. Two studies describe P2Y-mediated *c-fos* induction [50,52], with both using luciferase-based reporters to assess the induction of *c-fos* promoter elements. In UMR106 cells elevated Ca^{2+} was shown in response to co-stimulation with PTH and ADP and can activate the Ca^{2+} sensitive Ca/CRE promoter element to sufficiently drive *c-fos* gene expression [52]. On the other hand it was shown that in SaOS-2 cells, elevated cAMP arising from PTH stimulation can activate the Ca/CRE promoter element, although nucleotides had no impact on reporter activity [50]. In this study another element within the *c-fos* gene - serum response element was activated by nucleotide evoked Ca^{2+}. However, SaOS-2 cells didn't have the SRE-reporter construct and therefore, a rat derived cell line UMR106, which show similar behaviours were used as the basis for the reporter assay instead. Out of the nucleotides used in these studies (ATP, ADP and UTP), ADP along with PTH induced the greatest levels of *c-fos* [50,52].

Three further studies identify downstream signalling pathways associated with P2RX7. Firstly in TE85 OS cells transfected with P2RX7, Nuclear factor of activated T cells complex 1 (NFATc1) which is dependent upon intracellular Ca^{2+} mobilization had increased activation [59]. In MNNG-HOS cells western blotting showed P2RX7 activation with BzATP results in a 1.6-fold increase in Akt phosphorylation. Downstream of the Akt pathway is mammalian target of rapamycin (mTOR) which is also phosphorylated almost 2-fold, resulting in increased hypoxia-inducible factor 1-alpha (HIF1α) and vascular endothelial growth factor (VEGF) protein levels. Another key protein modulated by P2RX7 activation is GSK3β whose phosphorylation increases by 3.3-fold, resulting in elevated β-catenin and the nuclear transcription factor TCF-1. Thus, P2RX7 additionally regulates the Wnt pathway. Blocking the receptor with shP2RX7 resulted in a marked decrease in phosphorylation of these key signalling proteins [62]. Finally it has been shown that treatment with the P2RX7 antagonist A740003 downregulated *FN1/LOX/PDGFB/IGFBP3/BMP4* gene axis in transfected MNNG-HOS+P2RX7B cells using RNA-seq [63]. A summary of downstream pathways to P2 receptors in PBC is shown in Table 6. Although P2 receptor signalling can activate different pathways, they can also act as a downstream effectors and therefore be activated by other signalling and

Table 6. An overview of studies relating to signalling pathways associated with P2 receptors.

Study	PBC model	Non-PBC comparator or control	Therapeutic intervention (agonist or antagonist)	Pathway detection techniques	Main findings
Ca^{2+} signalling					
Kaplan *et al*., 1995 [43]	UMR106	MC3T3-El mouse osteoblast cell line, vehicle treated cells.	100 μM ATP, UDP, UTP, 2-MeSATP, 0.01 to 1 μM PTH	Fluorescent Ca^{2+} assay	PTH potentiates ATP or UTP-mediated Ca^{2+} signalling in UMR106 cells.
Bowler *et al*., 1999 [50]	SaOS-2	Primary HBDCs, vehicle control.	10 μM ATP, ATPγS, UTP, ADP, 100 ng/mL PTH	Fluorescent Ca^{2+} assay	PTH does not potentiate ATP or UTP-mediated Ca^{2+} signalling in SaOS-2 cells.
Buckley *et al*., 2001 [52]	UMR106	No cell comparator, vehicle control.	0.1 to 100 μM 2-MeSADP, ADP, ATP, UTP, 2-MeSATP, 100 ng/mL PTH	Fluorescent Ca^{2+} assay	PTH potentiates ADP-mediated Ca^{2+} signalling in UMR106 cells.
D'Andrea *et al*., 2008 [56]	SaOS-2, ROS17/2.8	HOBIT, vehicle control.	1 to 100 μM ATP, UTP, ADP, 10 μM to 1 mM H_2O_2	Fluorescent Ca^{2+} assay	H_2O_2 potentiates Ca^{2+} signalling through sensitisation of P2RY2 to nucleotides.
Liu & Chen., 2010 [58]	HOS	PC12, data presented as percentage of untreated control response.	1 to 1000 μM ATP, 100 μM BBP, 5 to 800 μM Suramin	Fluorescent Ca^{2+} assay	Phthalates supresses Ca^{2+} influx associated with P2X receptors.
Upregulated signalling pathways					
Bowler *et al*., 1999 [50]	SaOS-2 UMR106	Primary HBDCs, vehicle control.	10 μM ATP, ATPγS, UTP, ADP 100 ng/mL PTH	Northern blotting, Luciferase reporter gene assay	*c-fos* is induced when PTHr and P2 signalling are activated.
Buckley *et al*., 2001 [52]	UMR106	No cell comparator, vehicle control.	0.1 to 100 μM 2-MeSADP, ADP, ATP, UTP, 2-MeSATP 100 ng/mL PTH	Luciferase reporter gene assay	*c-fos* is induced when PTHr and P2 signalling are activated.
Giuliani *et al*., 2014 [59]	TE85	Jurkat cells, vehicle and untreated control.	50 μM BzATP 10 μM cyclosporin	NFATc1 activation assay (ELISA-based)	NFATc1 transcription factor activation in response to BzATP activation of P2RX7.
Zhang *et al*., 2019 [62]	MNNG-HOS	Human BMSCs; untreated control; vector control.	5 to 125 μM BzATP 5 μM A740003 Lentiviral infection	WB, ELISA	BzATP-mediated P2RX7 activates Pi3K/Akt, mTOR/HIFa/VEGF and Wnt pathways.
Tattersall *et al*., 2021 [63]	TE85, MNNG-HOS	Untransfected cells, HEK-293.	10 μM A740003	RNA-seq	A740003 treatment downregulated *FN1*/*LOX*/*PDGFB*/*IGFBP3*/*BMP4* in P2RX7B transfected MNNG-HOS cells.
P2 receptors downstream of other signalling					
He *et al*., 2020 [67]	Syngeneic mouse NCTC 2472	Sham mice.	A317491 (20 mg/10 μL) anti-Ryk (50 ng/10 μL) Wnt5b (50 ng/mL)	WB, ELISA	P2RX3 can be upregulated by Wnt5b/Ryk.

influence the bone-tumour microenvironment. This was the case in one study *in vivo*. Wnt5b/Ryk signalling was shown to induce the activation of CaMKII in DRG neurons which was supressed when pre-treated with anti-Ryk antibody. Wnt5b/Ryk was then shown to increase P2RX3 expression in the neurons and was attenuated when using a CaMKII inhibitor. Therefore, it was determined that Wnt5b can promote P2RX3 membrane trafficking via CaMKII in DRG neurons, the use of the inhibitor in mice with PBC was then shown to improve PBCP assessments [67] and therefore suggests that targeting P2RX3 within the bone-tumour microenvironment as opposed to the primary tumour could potentially reduce pain.

3.6.6 The Functional Role of P2 Receptors in PBC

3.6.6.1 Effects of P2 Receptors on Proliferation in PBC. Five studies show that P2X receptors can play a role in proliferation. When stimulated with 100 μM extracellular ATP or ATPγS for 24 hours MG-63 cells activate DNA synthesis which was shown through [3H] thymidine incorporation and resulted in increased cell proliferation. This was then confirmed by actual cell count and was in parallel with elevated Ca^{2+}. Based on agonist and antagonist effects, such as no effect with UTP or UDP inducing DNA synthesis and also PPADS but not reactive blue 2 inhibiting DNA synthesis, it was indicated that P2X receptors and not P2Y receptors were responsible for this effect [51], but the actual receptor subtype was not confirmed. In HOS cells ATP exposure for 40 hours could both increase and decrease cell proliferation dependent upon concentration, where $\leq$ 100 μM increases cell proliferation and $>$200 μM decreases it. The effect on proliferation was attributed to P2X receptors as 100 μM UTP and UDP did not affect cell proliferation and the increase was suppressed by BBP which antagonises P2X receptors. Of the subtypes, only P2RX4 and P2RX7 were shown to be expressed in these cells, but it was not distinguished which of the two P2X receptors was responsible for increasing proliferation [58].

P2RX7 and its splice variants have been shown to affect proliferation [59,62,63]. Transfection of TE85 with the full length P2RX7A, P2RX7B or cotransfection of P2RX7A and P2RX7B all provided a strong growth increase compared to control TE85 cells over a 72-hour period. Additionally after 24 hours, treatment with 100 μM BzATP further increased proliferation, whilst this was attenuated with 100 μM A740003 or 4U/mL Apyrase. P2RX7B, as opposed to P2RX7A alone or P2RX7A and P2RX7B together, results in the greatest levels of cell proliferation. Further to this, proliferation was assessed in OS patient samples where a double IHC staining was used, Ki67 staining to show proliferation and N and C-terminal specific antibodies to determine receptor isoform expression. Tissue solely expressing P2RX7B were shown to have increased tumour cell density in clinical samples [59].

In a further study, MNNG-HOS and SaOS-2 cell proliferation was detected using a CCK-8 assay over a 72-hour period [62]. When stimulated with 5, 25 or 125 μM BzATP proliferation was increased and was attributed to P2RX7, as 5 μM A740003 and shRNA targeting P2RX7 both reduced this effect. These findings were further shown *in vivo*, where 2.5 mg/kg BzATP increases tumour volume compared to placebo treated mice and 0.025 mg/kg of A740003 causes a reduction compared to control mice as measured by callipers. The same effect was also shown when the primary tumour was stained for Ki67 where BzATP treatment increased the number of proliferating cells and A740003 reduced this effect [62]. Finally, further evidence for the role of P2RX7B in OS proliferation has been shown, with transfection of P2RX7B into both TE85 and MNNG-HOS OS cells resulting in increased proliferation over a 7 day period which was reduced following treatment with 100 μM A740003 [63].

3.6.6.2 Effects of P2 Receptors on Mineralisation in PBC. Only one study associates P2 activity with bone mineralisation, this was assessed over a 21 day period by Alizarin red staining in TE85 OS cells. Transfection of P2RX7A, P2RX7B and both isoforms together in cells confers differing effects. The P2RX7A did not affect bone mineralisation, P2RX7B reduced mineralisation and P2RX7A and P2RX7B together increased mineralisation. This was attributed to differences in spontaneous ATP release and ability to form a pore. Typically, P2RX7A form pores whilst P2RX7B lacks the pore forming c-terminal, however in Te85 cells expression of both P2RX7A and P2RX7B together was required to induce pore formation, potentially due to the isoforms co-associating on the membrane [59]. Due to the pore forming ability of P2RX7A and P2RX7B together, there is greater spontaneous ATP release which can drive mineralisation. This is important as OS patients often present with large amounts of ectopic mineralised bone, and this suggests that P2RX7 isoform expression can contribute towards this phenotype. Furthermore, the cells expressing the P2RX7B isoform alone which lacks the pore formation ability is suggestive of an undifferentiated state compared to cells expressing both variants that allow for pore formation. This, therefore, can inform on the aggressiveness of the cancer and help identify which patients may benefit from targeting P2RX7 [59].

3.6.6.3 Effects of P2 Receptors on Cytotoxicity in PBC. Three studies demonstrate an anti-tumour effect associated with P2 receptors. The first evidence for the direct involvement of P2RX7 in eliciting cytotoxic effects was in SaOS-2 cells [53]. BzATP treatment resulted in lactate dehydrogenase (LDH) release and membrane blebbing characteristic of apoptosis, this was further confirmed with TUNEL staining. Pre-treatment with the P2X antagonist PPADS prevented the blebbing and reduced TUNEL staining to levels comparable to untreated controls [53]. It was then further

shown by using an MTT assay that ATP at >200 μM results in reduced cell viability, but the study did not confirm which specific receptor resulted in this effect [58]. P2RX7 has also been shown to be indirectly involved with cytotoxicity in U2OS OS cells, as activation of P2RX7 with ≥ 100 μM ATP resulted in the opening of methotrexate permeable channels and cell death [60].

3.6.6.4 Effects of P2 Receptors on Metastasis in PBC. Two studies showed that P2RX7 and its splice variants are involved in metastasis [62,63]. *In vitro* wound-healing, transwell invasion [62,63] and cell adhesion [63] assays were used to show the involvement of P2RX7 and P2RX7B signalling in the migration and invasion of MNNG-HOS, TE85 [62,63] and SaOS-2 cells [62]. Full length P2RX7 or the truncated P2RX7B both provided a strong increase in migration, invasion and decreased cell adhesion. As with increased proliferation, these cell behaviours are dependent upon the PI3K/Akt pathway where activation of P2RX7 is further associated with epithelial-to-mesenchymal transition (EMT), whereby E-cadherin is diminished at the mRNA and protein levels whilst EMT markers such as vimentin, fibronectin and SNAIL are elevated [62]. When targeting the P2RX7 *in vivo*, mice treated with BzATP showed a greater number of metastatic nodules in the lung when compared to the controls, which could be reduced in mice treated with A740003 [62]. Further to this P2RX7B transfected MNNG-HOS cells tended to have increased lung metastasis in both a xenograft and tail vein models [63].

3.6.6.5 Effects of P2 Receptors on PBC Pain. Three studies demonstrate the involvement of P2RX3 in PBCP [64, 66,67]. Firstly it was shown *in vivo* that A317491 reduced OS-induced thermal hyperalgesia [64] and that anti-P2RX3 antibody treated mice showed reduced skin hypersensitisation associated with PBCP, but did not have improvements in the overall skeletal pain behaviours [66]. Further to this P2RX3 was upregulated in the DRG neurons in the bone-tumour microenvironment by Wnt5b acting through CaMKII in bone tumour bearing mice, this upregulation resulted in increased thermal hyperalgesia and PBCP, which was reduced with A317491 [67]. The only other P2 receptor assessed for its role in PBCP is the P2RX7. It was demonstrated that P2RX7 KO mice had PBCP behaviours with a more severe phenotype and earlier onset compared with wild-type tumour bearing mice [65].

4. Discussion

The role for purinergic signalling involving both P2Y and P2X receptors in the tumour microenvironment and their contribution towards the pathogenesis of many cancers is now acknowledged due to the growing evidence from *in vitro* and *in vivo* studies, and have promising therapeutic implications. The aim of this systematic review was to assess the role of P2 receptors specifically in PBC relating to subtype expression, associated signalling pathways and the functional consequences associated with each individual receptor (summarised in Fig. 2). The majority of papers included in this systematic review focus on OS cells however, many of the early studies use these cells as a replacement for osteoblasts to investigate purinergic signalling in normal bone physiology. This is a huge drawback causing a lack of focus on OS as a disease state, with some observed effects not being attributed to OS but to osteoblast behaviour with no particular focus truly on PBC. A further criticism is that OS cell lines can provide different models when cultured or used in mice and can be of murine or human origin, the human cell lines would have more relevant translatable biology relating to P2 receptors. SaOS-2 cells are largely characteristic of mature osteoblasts. The MG-63 population comprise cells with both mature and immature osteoblastic features and U2OS cells are described as osteoblastic whilst sharing fibroblastic features [68]. MNNG-HOS cells are derived from TE85 cells and form tumours in mice which produce a phenotype with large amounts of ectopic bone [69] which represents clinical OS but demonstrate very little metastasis [69,70]. In some instances, the exact receptor profiles within certain cell lines has been disputed, this can be partially accredited to differences in methodology, but also there is the possibility for long term culture to alter cell phenotype. A shift towards the use of tissue from PBC patients would be beneficial as only a minimal number of studies used PBC tissue with low sample sizes. This review also includes *in vivo* experiments utilising human xenograft and syngeneic murine models. The use of syngeneic models gives the added benefit of an intact immune system however a xenograft uses human cells [62–67], patient-derived xenograft (PDX) models could be used as an alternative which would provide the most relevant model to study P2 receptor biology in PBC due to their physiologically relevant tumour microenvironment, heterogeneity and natural tumour progression.

4.1 P2Y Receptors in PBC

4.1.1 P2RY1 and P2RY2

Early studies within this systematic review focussed on P2Y receptors and historically these were detected first. Molecular characterisation was not provided in some studies and therefore they depended on Ca^{2+} signalling meaning the specific subtype is not demonstrated. Of the P2Y subfamily, P2RY1 and P2RY2 are the most studied within this systematic review, with expression having been shown in murine and human OS cell lines in addition to GCTB tumours isolated from patients. The studies focus on expression and signalling but do not assess functionality or include animal models despite P2RY1 activation been previously shown to induce apoptosis and reduce proliferation in prostate cancer [71] and P2RY2 promoting migration and invasion in breast cancers [72]. P2RY1 and P2RY2 were in-

vestigated in GCTB but samples were taken from only three patients [44,49,50]. In OS cell lines the studies showed interactions with reactive oxygen species and systemic factors such as PTH in mediating Ca^{2+}signalling. Previous investigations studying PTH in OS have shown that it is upregulated and correlates with poor prognosis, tumorigenesis, chemoresistance and metastasis [73] and therefore the relationship between PTH and P2 signalling could provide a novel target for treatment. Further to this, downstream of Ca^{2+}signalling, *c-fos* induction has been reported in response to both P2RY1 [52] and P2RY2 [50], in non-malignant osteoblasts, upregulation of *c-fos* has been shown to regulate proliferation and differentiation during development [74] whilst *c-fos* overexpression in bone is known to cause transgenic mice to develop OS [75]. Therefore, further investigations of P2Y induction of *c-fos* and its associated signalling could potentially identify novel therapeutic targets in OS.

4.1.2 P2RY11

Evidence relevant to P2RY11 in PBC showed that this receptor was expressed at both mRNA and protein level in SW1353 chondrosarcoma cells [61] and inhibition of P2RY11 using NF157 resulted in reduced expression of TNF-α-induced MMP and ADAMTS, enzymes which contribute towards the breakdown of the articular cartilage extracellular matrix. However, a strong criticism of this study was that it was performed in the context of osteoarthritis and whether this receptor affects chondrosarcoma progression or metastasis from a tumorigenic perspective was not explored. Further to this no *in vivo* data was presented. In other cancers, the role of P2RY11 is gradually developing. P2RY11 was shown to be highly expressed in hepatocellular carcinoma tissue compared to normal tissue and promotes migration *in vitro* [76], this was similarly the case in breast cancer where siRNA knockdown of P2RY11 inhibited cell migration, as did NF157 which reduced breast cancer cell migration in a dose-dependent manner [77]. P2RY11 expression and function has not been explored in any other PBC models and therefore it could provide a novel target for OS or Ewing's sarcoma where NF157 could be used.

In summary, P2YR expression in PBC remains relatively underexplored and represented a gap in knowledge, given that some P2Y targeted treatments (such as the P2RY12 antagonist Clopidogrel) have achieved significant successful clinical use for other conditions [78] targeting them in a PBC context may be beneficial.

4.2 P2X Receptors in PBC

4.2.1 P2RX7 and P2RX4

Several studies included within this systematic review focus on P2RX7 in OS with expression and function described in multiple cell lines *in vitro*, *in vivo* and in clinical patient samples. Evidence from this review suggests a pro-tumour role for P2RX7 in OS mediated by its ion channel, and therefore its inhibition could be of therapeutic value, whilst its pore activation could also induce cell death. The dual nature of P2RX7 in OS is still being established. High levels of P2RX7 was demonstrated in patient samples [59,62] and this could be expanded by linking disease characteristics such as stage or presence of metastasis in order to identify specific cohorts of eligible patients that may be suitable for P2RX7 targeted treatments. Further to this, P2RX7 activation has been associated with signalling pathways such as NFATc1 [59], Pi3K/Akt, Wnt and mTOR/HIF1α/VEGF [62] which are responsible for proliferation and tumour progression. Wnt signalling has other implications in governing bone homeostasis, where it has been shown to control the balance between osteoblasts and osteoclasts [79] and altered mTOR signalling in OS patients is linked with poor prognosis [80]. This provides evidence that P2RX7 directly regulates oncogenic pathways within OS, thus highlighting the receptor as a potential target for therapeutic inhibition. Furthermore, the metastatic potential in relation to P2RX7 in OS has also been demonstrated [62] where P2RX7 was shown to promote both migration and invasion *in vitro* and lung metastasis *in vivo* again linking to progression and disease severity, it also suggests that targeting metastatic patients may be beneficial. This systematic review also provides evidence for an anti-tumour role associated with P2RX7. Firstly, activation of the P2RX7 pore can increase chemotherapy uptake and promotes cytotoxicity [60] and activation of the P2RX7 pore with BzATP results in apoptotic cell death [53]. Thus, exogenous activation of P2RX7 may also provide a reasonable therapeutic approach, however, not all P2RX7 isoforms have this ability and therefore only specific patient cohorts expressing non pore forming P2RX7 variants may benefit from therapeutic targeting of this receptor. There are limited studies regarding P2RX7 isoforms, nevertheless P2RX7B expression was shown to correlate to high cell numbers in OS patient samples, and provide the strongest growth stimulus to TE85 OS cells *in vitro,* when compared to other splice variants [59]. P2RX7B has further been suggested as a target in OS as expression of P2RX7B conferred a growth advantage to both TE85 and MNNG-HOS cells that could be reduced with A740003, a loss of cell adhesion and increased migration and invasion were also demonstrated suggesting P2RX7B may contribute towards a metastatic phenotype. This was further demonstrated *in vivo* in both a paratibial and tail vein model where P2RX7B expression had a tendency to increase incidence of metastasis to the lungs again supporting targeting metastatic OS patients. When inhibiting P2RX7B, A740003 downregulated the *FN1*/*LOX*/*PDGFB*/*IGFBP3*/*BMP4* gene axis, which are all known to promote cancer progression and thus could provide further therapeutic targets individually, or as combination treatments [63]. A final splice isoform P2RX7K has been suggested to be involved in bone re-

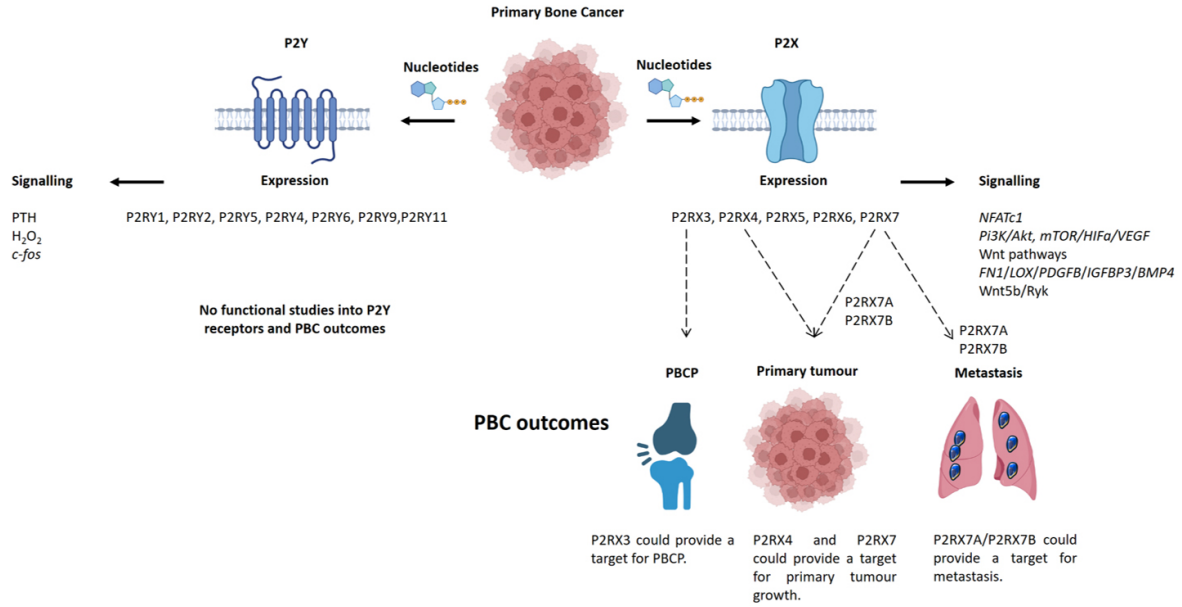

Fig. 2. Schematic representation of P2Y and P2X expression, signalling and function in PBC and the potential PBC outcomes that could be influenced and targeted.

modelling in murine models as KO mice had differences in skeletal phenotypes [65]. Despite these studies, the expression, signalling and role of other functional and non-functional P2RX7 splice variants or loss and gain of function polymorphisms within PBC has not been explored. This is important as P2RX7 demonstrates a bifunctional phenotype amongst other cancer types, with its function determining a pro or anti-tumour nature. Therefore, regarding P2RX7 targeting in PBC, the specific variant for individuals must be considered. Additionally, the role of P2RX7 in other PBCs has also yet to be explored and could be exploited in Ewing's sarcoma, chondrosarcoma, or chordoma. In addition to targeting P2RX7, some studies in this review demonstrate a high expression of P2RX4 in various OS cell lines, however, the signalling and function associated with its expression is absent and has not been researched further. One study did suggest that both P2RX4 and P2RX7 play a role in proliferation of OS cells [58]. P2RX4 and P2RX7 heterotrimeric receptors have been identified [81] with their role in breast cancer previously shown where P2RX4/P2RX7 pannexin-1 channels induce a change from pro-survival functionality to an induction of mixed apoptotic and necrotic cell death, suggesting that P2RX4 activation could reduce tumour growth [82]. This could be due to P2RX4 mediating the formation of a transmembrane pore which increases permeability to larger molecules in a similar but not identical way to P2RX7 [83]. It has also recently been shown that targeting P2RX4 can suppress prostate cancer growth both *in vitro* and *in vivo* [84] and therefore studies in different cancers are indicating P2RX4 as a promising therapeutic target. Further to this P2RX4 antagonists such as 5-BDBD, NP-1815-px, PSB12062 BX430 and BAY-1797 are in development for a number of conditions [85] and could be available for assessment of anticancer effects in PBC. Interestingly P2RX4 has also been linked to cancer pain [86] which has yet to be explored in PBC and could further play a similar role that of P2RX3. Overall, targeting P2RX7 and P2RX4 either individually or together could provide a novel therapeutic target in OS and other PBCs.

4.2.2 P2RX3

PBCP is a debilitating symptom associated with PBC, evidence from this systematic review demonstrates that pharmacological inhibition of P2RX3 in OS using A317491 or anti P2RX3 antibody *in vivo* is a potential therapeutic for pain reduction [64,66,67] acting within the bone-tumour microenvironment. This is important as managing PBCP would be beneficial to PBC patients when approaching their treatment regimen. The role of P2RX3 in cancer pain has previously been described [87] and ATP in the tumour microenvironment can potentially activate nociceptive nerve endings responsible for the pain response, therefore, inhibition may be beneficial [87]. Further to this P2RX3 inhibitors are now approved for human use where Gefapixant is used clinically for chronic cough [88] and therefore drugs that target P2RX3 are available for repurposing. Aside from its role in pain, P2RX3 has not been explored regarding its effect on the primary tumour itself, or in any other PBC type such as Ewing's sarcoma or chondrosarcoma and could be a possible target, and further studies are therefore warranted to elucidate the therapeutic potential of P2RX3 in PBCs.

4.2.3 Summary

PBC is a rare and complex disease with high levels of heterogeneity and therefore there has been a lack of new

therapeutics developed, this review aimed to systematically highlight progress in the purinergic field concerning which P2 receptors could potentially play a role in PBC progression or associated symptoms and which could provide suitable targets. Based on the evidence in this systematic review, there is a lack of progress in P2Y receptor characterisation and function in OS, aside from early studies. The most promising therapeutic targets we believe are P2X receptors including P2RX3 for PBCP and P2RX4/P2RX7 for targeting the primary tumour, as these have been shown to contribute towards important OS outcomes *in vitro* and *in vivo* such as growth and metastasis. It may be the case that only certain cohorts of patients would benefit from this approach based on the individuals' expression profile of specific receptors or their isoforms. Therefore, future therapeutic strategies will benefit from a comprehensive characterisation and association of P2 receptors with clinical outcomes of patients with PBC.

Author Contributions

AG designed the research study, LT, DCG, VLT, KMS, NBAL performed the research, LT, DCG, analysed the data, LT, DCG, AG wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

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