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Yet, perhaps it was too narrow a selection for a conference topic. A wider and more diverse audience would have been attracted by the addition of, for example, a dedicated session on ion channels (a disclaimer: this is a topic relevant to the author's own research). Ion channels are, after all, the most rapidly acting of signaling entities, and with over 400 known genes, they are running a close second to the protein kinases. New opportunities for automated high-throughput screening of ion channels are also emerging, and both the advantages and disadvantages of these techniques deserve dissemination to a wider audience. The advent of diagnosis and targeted chemotherapy by nanotechnology was another missing topic that could have been useful to include in this conference. Hopefully, the organisers will be willing to revisit this important subject of signal transduction as targets of drug development in a more expansive manner. Although the conference was definitely rewarding, it was ultimately a frustratingly short day-and-a-half.

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Osteolytic bone diseases: physiological analogues of bone resorption effectors as alternative therapeutic tools

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The treatment of osteolytic diseases has relied predominantly on the use of bisphosphonates. Although the efficacy of bisphosphonate treatment in inhibiting bone resorption has been clearly demonstrated, several secondary and undesirable side-effects have been also reported. In this context, alternative treatments to bisphosphonate therapy, based on the knowledge of osteoclast biology, have been proposed. Bone resorption is tightly regulated by numerous factors including hormones, cytokines and integrins. Among these cytokines, the OPG–RANK–RANKL molecular triad, three members of the TNF cytokine/receptor family, is key in regulating osteoclast activities. Similarly, αvβ3 integrin allows the binding of osteoclasts on calcified tissues. Thus, cytokines, their signaling and integrins represent new targets to treat osteolytic diseases and here we describe new alternate strategies for the treatment of osteolysis.

Bone is a specialized connective tissue formed by mineralization of an organic matrix that confers its elastic and strength properties. Bone remodelling allows the skeleton to adapt to mechanical constraints and maintains phosphocalcic homeostasis through coordinated phases of formation and resorption. Thus, bone remodelling involves the synthesis of organic matrix by osteoblasts and bone resorption by osteoclasts. The equilibrium between osteoblastic and osteoclastic activities is tightly regulated by numerous extracellular molecules (cytokines, hormones and vitamins), which interact with membranous and/or nuclear receptors, thus inducing intracellular signalling. Among these molecules, the Tumor Necrosis Factor (TNF) and TNF-receptor family is particularly implicated [1]. Any disturbance in the equilibrium of osteoblast–osteoclast activities leads to the development of bone pathologies. Thus, increased osteoclast activity is observed in many osteopathic disorders, including postmenopausal osteoporosis, Paget's disease, primary bone tumors, lytic bone metastases, multiple myeloma, rheumatoid arthritis or aseptic prosthesis loosening, leading to increased bone resorption, hypercalcemia and a loss of bone mass [2,3]. In some rare cases, tumour development leads to osteoformation without osteolysis, as in some forms of osteosarcoma or osteoblastic metastasis predominating in patients with prostatic adenocarcinoma. In most cases, the skeletal manifestation of malignancy is focal osteolysis. This imbalance in favour of bone resorption can result from the acquisition of new cellular properties by bone cells: increase in the proteolytic activity, alteration in local or humoral factors expression. Tumour products can either stimulate

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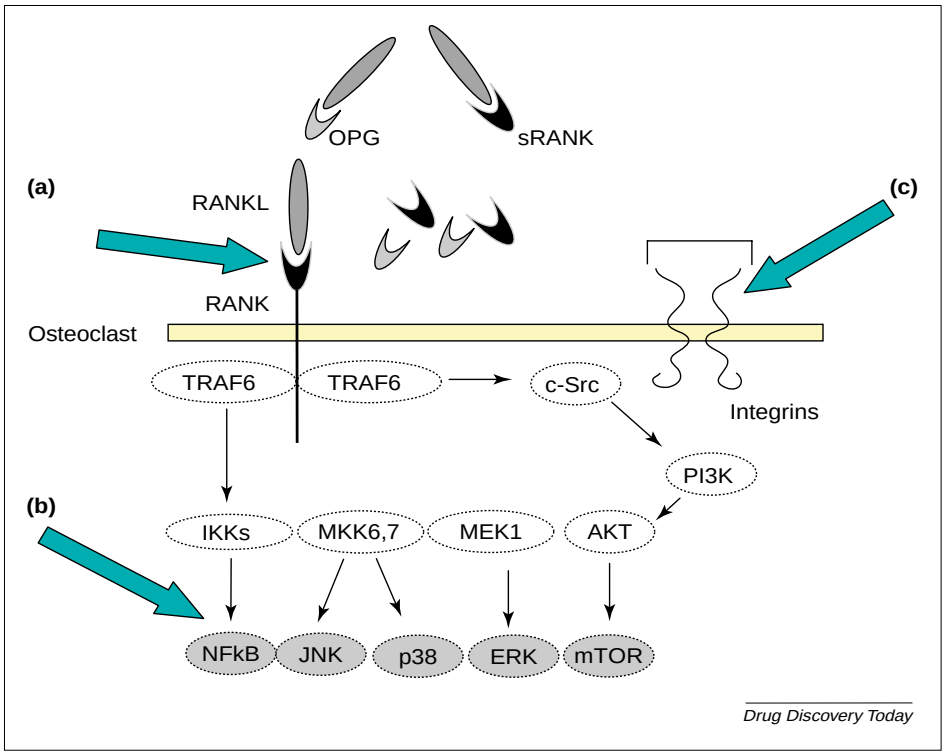

FIGURE 1

New therapeutic strategies of osteolytic bone diseases targeting key molecular effectors of bone resorption. Bone remodelling involves synthesis of organic matrix by osteoblasts, and bone resorption by osteoclasts. Bone resorption is tightly regulated by hormones and cytokines through direct or indirect action on osteoclasts and by numerous interactions with extracellular matrix components through integrin expressed on osteoclast membrane. OPG–RANK–RANKL molecular triad, three members of the TNF cytokine/receptor family is key triad regulating osteoclast activities. Similarly, αvβ3 integrin allows the binding of osteoclasts on calcified tissues. In this context, both cytokines and their intracellular mediators and integrins represent strategic targets to treat osteolytic diseases. These new therapeutic approaches are based on molecules blocking RANKL activities or osteoclast adhesion to extracellular matrix using: **(a)** recombinant OPG, soluble RANK or peptidomimetics; **(b)** specific RANKL signalling inhibitors, that is, inhibitors of NF-κB, a key transcription factor of RANKL signaling; **(c)** peptides blocking osteoclast integrin interactions to the extracellular matrix

osteoclast formation locally in the bone microenvironment or systemically through production of hormones, such as parathyroid hormone-related protein (PTH-rP), the mediator of the humoral hypercalcaemia of malignancy. Other factors produced by tumour cells that can stimulate osteoclastic bone resorption include interleukin-1 (IL-1), IL-6, tumour necrosis factor-α (TNF-α), macrophage inflammatory protein-1-α (MIP-1α) [4] and Receptor Activator of Nuclear Factor-Kappa B Ligand (RANKL) [5,6]. These agents, released into the bone microenvironment, act on osteoblastic stromal cells to enhance the production of osteoclast activating factors. These agents, released into the bone microenvironment, act on osteoblastic stromal cells to enhance the production of osteoclast-activating factors.

To date, the treatment of osteolytic diseases has relied predominantly on the use of bisphosphonates. Although the efficacy of bisphosphonate treatment in inhibiting bone resorption has been clearly demonstrated, several secondary and undesirable side-effects have been reported. Thus, alternative treatments to bisphosphonate therapy, based on the knowledge of osteoclast biology (Figure 1), have been proposed.

Bisphosphonates: the standard therapeutics for the treatment of osteolytic bone disease

Bisphosphonates are stable synthetic analogues of the naturally occurring pyrophosphate molecule that are resistant to enzymatic hydrolysis induced by osteoclasts [7]. Different side chains can be added to the central carbon atom to produce a range of bisphosphonates with varying clinical activity and potency [8]. Bisphosphonates (BP) can be broadly divided into non–nitrogen-containing and nitrogen-containing bisphosphonates. Among them, zoledronic acid which contains a second nitrogen atom in the imidazole ring, is one of the most potent bisphosphonate known to date [9].

Bisphosphonates have proven to be effective inhibitors of bone resorption, particularly when given intravenously. They accumulate in the mineralized bone matrix, preventing the osteoclastic resorption of this matrix [10]. Although their inhibitory effects on bone resorption can be, in part, attributed to their physicochemical properties, studies are now beginning to elucidate their molecular mechanisms of action [8–14]. Studies now show that when BP are released during the process of bone resorption and are internalized by osteoclasts, they inhibit the formation and osteolytic activity of osteoclasts and reduce their survival [15]. In addition, BPs also indirectly inhibit the activity of osteoclasts by modulating signaling from osteoblasts to osteoclasts [16].

The administration of BP have been shown to normalize serum calcium, reduce the incidence of skeletal complications and can reduce the need for radiation and surgery to bone. In addition, bisphosphonates appear to reduce the skeletal tumor burden in animal models by making the bone microenvironment a less favorable site for tumor-cell growth [9].

Although BPs are effective and generally well-tolerated when used to treat, for example, hypercalcemia of malignancy and periprosthetic bone loss [9,17–21], undesirable side-effects have been reported. Thus, alendronate acts on monocytes' antigen-presenting/accessory cell functions, which then could reduce the immune response [22]. Intravenous injection is frequently associated with an increase in body temperature and an influenza-like syndrome. BPs have been also associated with nephrotoxicity [23] and to severe hypocalcaemia after being given intravenously [24]. Moreover, White *et al.* recently described a case of bisphosphonate-induced osteopetrosis in a 12-year old boy, demonstrating that acquired osteopetrosis or marble bone disease could therefore result from treatment with bisphosphonates [25]. These authors then

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cautioned that excessive doses of bisphosphonates might compromise skeletal quality in growing patients, despite a concomitant increase in bone density.

Alternative therapeutic approaches of bisphosphonates

Bone cell activities are tightly orchestrated by a complex molecular network that serves to maintain bone integrity throughout life. This extracellular network modulated by several cytokines controlling osteoblastic differentiation as well as osteoclastic bone resorption. Any disturbance between these effectors leads to the development of skeletal abnormalities, characterized by decreased (osteoporosis/osteopenia) or increased (osteopetrosis) bone mass. Most notable of the effectors involved in the bone resorption–bone apposition equilibrium is RANKL, which is a member of the TNF cytokine family [1]**.** Indeed, RANKL is the main regulator of osteoclast differentiation. Within the bone system, soluble- and membrane-bound forms of RANKL expressed by osteoblasts, exert their activities through binding to their receptor, RANK, on osteoclasts. The third protagonist, osteoprotegerin (OPG) produced by osteoblasts, acts as a decoy receptor for RANKL, preventing it from binding to and activating RANK. It also inhibits the development of osteoclasts and downregulates the RANKL signalling through RANK. Any disturbance in the OPG–RANKL equilibrium leads to the development of bone pathologies [1,26].

Bisphosphonates have been demonstrated to act on OPG and RANKL production by human osteoblast-like cells. Pamidronate and zoledronate stimulate OPG production by primary human osteoblasts [27], and zoledronate influences RANKL expression in human osteoblast-like cells by activating TNF-α-converting enzyme (TACE) [16]. These findings show that bisphosphonates, in addition to exerting a direct effect on bone and tumor cells, act on bone resorption through the modulation of the OPG–RANK–RANKL equilibrium.

Recombinant OPG and RANK as therapeutic agents

In light of the data described above, novel strategic treatments for bone loss are emerging from an understanding of the functional status of the RANKL axis. In this context, the use of recombinant OPG or any other molecule able to block the interaction between RANKL and RANK presents a major therapeutic interest. Indeed, OPG (50 mg/kg three times per week for 4 weeks, i.v.) reverses osteoporosis**,** prevents bone erosion in arthritis and blocks cancer-induced skeletal destruction [1,28]. Other data revealed that OPG exhibits hypocalcemic effects in normal mice and in hypercalcemic nude mice bearing tumors and was also associated humoral hypercalcemia of malignancy. OPG also inhibits osteolysis, increases survival in a murine model of multiple myeloma and inhibits growth development in a prostate cancer model [1,28]. Two clinical trials using a genetically engineered form of OPG, termed OPG-Fc, were conducted in postmenopausal women [29] and in patients suffering from multiple myeloma or breast carcinoma with osteolytic lesions [20]. Moreover, results from these studies indicated that OPG-Fc injections were well tolerated and revealed the efficacy of a single s.c. injection of OPG-Fc (0.3 mg/kg or 1 mg/kg), which rapidly and profoundly reduced bone resorption and bone turnover as indicted by the decrease of bone resorption markers (urinary NTX/creatinine). These effects were comparable with those obtained with pamidronate. However, although the first reports of the effectiveness of OPG-Fc were promising, the ability of OPG to bind TNF Related Apoptosis Inducing Ligand (TRAIL) proved controversial in the case of tumor-associated osteolysis. Indeed, the binding of OPG to TRAIL blocked the TRAIL-mediated apoptosis of sensitive cancer cells [31] and is a survival factor of cancer cells [32].

To overcome this problem, the ability of a RANK-Fc fusion molecule has been evaluated as a potential therapy to prevent bone resorption associated or not to tumor progression [33,34]. To this end, RANK-Fc was found to decrease the progression of prostate cancer in bone through inhibition of bone resorption (200 μg/mice i.p., three time per week for 6 weeks) [35]. Moreover, the effect of RANK-Fc was transient as revealed in a fracture healing model. Indeed, repeated administration of high dose of RANK-Fc (10 mg/kg i.p.) reduced temporarily the osteoclast number, which returned to baseline once injections were discontinued [36]. Thus, osteoclastic depletion via inhibition of RANK signaling (RANK-Fc) is a viable option for the treatment of pathological bone loss. The main advantage of such a strategy is that RANK-Fc does not block TRAIL-mediated apoptosis while it specifically inhibits osteoclastogenesis through RANKL.

Recently, Amgen group produced a fully human monoclonal antibody to RANKL called AMG 162 [36], which was evaluated in 49 postmenopausal women (randomized, double-blind, placebo-controlled, single-dose, dose escalation study: 0.01 mg/kg to 3.0 mg/kg s.c.). The results demonstrated that AMG 162 was well tolerated and resulted in a dose-dependent rapid and sustained decrease from baseline in bone turnover, demonstrating that anti-RANKL antibody could be a convenient treatment for osteoporosis [37].

Peptidomimetics: agonist or antagonist peptides of bone effectors

Recent advances in computer modeling have enabled workers to model proteins using constructs obtained from their crystallographic coordinates or from homologous proteins of the same family (Figure 2). Models are then usually optimized using energy minimization and molecular simulation calculations and provide a model of putative ligand-receptor interaction sites, which are used to design small peptidomimetics that interfere with these sites. This strategy has already been used successfully to produce peptides able to block, for example, the erbB receptor, the EPO receptor or the renin–angiotensin system. Similarly, a peptidomimetic antagonist of the αvβ3 integrin inhibits bone resorption *in vitro* and prevents osteoporosis *in vivo* [38]. This integrin receptor binds to the Arg-Gly-Asp (RGD) sequence expressed by several extracellular matrix proteins. Moreover, osteoclast αvβ3 integrin interacts with RGD within bone matrix protein, mediating bone resorption. αvβ3 antagonists inhibit bone resorption by decreasing osteoclast bone resorptive activity or efficiency but not by inhibiting osteoclast adhesion to mineralized matrix [39]. More recently, Harms *et al.* demonstrated that an antagonist of the αvβ3 integrin suppressed the development of osteolytic breast cancer metastases

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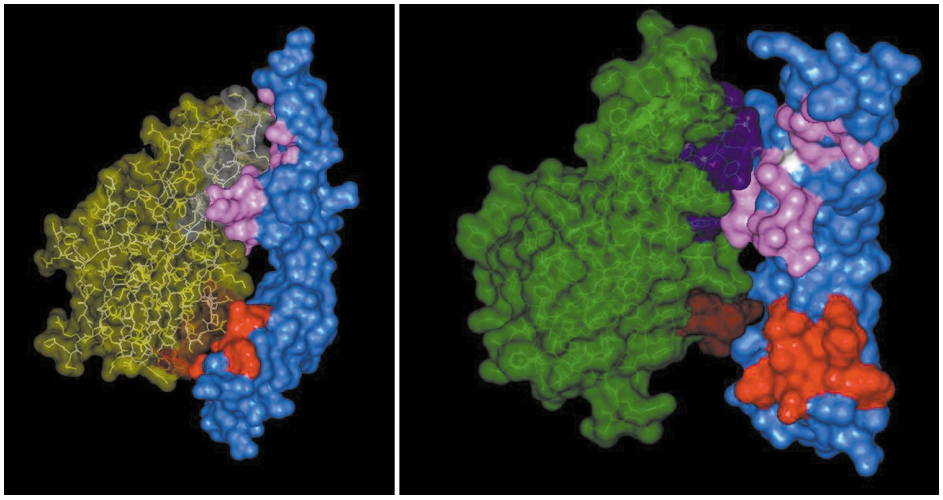

FIGURE 2

Interactions between the TNF and TNF receptor families. Surface representation of the TRAIL-DR5 complex using models constructed from crystallographic coordinates (1D4V, [26]). Several contact sites have been identified based on the structural analysis performed using *Insight II* (Accelerys Inc.,Cambridge, UK) and optimized using energy minimization and molecular simulation calculations. Thus, in this example, interaction of the monomer S of DR5 in cyan and the monomer A of TRAIL in light green show two preferential interaction sites colored in orange and in rose **(a).** Similarly, interaction of the monomer S of DR5 in cyan and the monomer B of TRAIL in green show two other preferential interaction domains colored in red and in violet **(b)**

(IC_{50} 0.2 nM, s.c.; no toxicity at a dose up to 1000 μM) and could inhibit the vicious cycle established between tumor cells and osteoclasts during bone metastase development [40]. The TNF-α receptor family is a privileged family based on their role in bone biology [41]. More recently, in the case of the RANKL axis, Cheng *et al.* reported a rationally designed small molecule that mimics osteoprotegerin [42]. In this interesting study, unlike soluble OPG that precludes the RANKL interaction with RANK, the small peptide called OP3–4 modulated RANK signalling, thus altering the biological function of RANK and facilitated the formation of a defective receptor complex by acting like a spacer between RANK and RANKL. OP3–4 inhibited osteoclastogenesis and total bone loss in an ovariectomized mouse model. Thus, OP3–4-treated mice exhibited an increase in total bone density in femur compared with the control group after 28 day treatment with 2 mg/kg/day s.c. (optimal dose: 6 mg/kg/day; IC_{50} 10μM). These pharmaceutical designs led to the synthesis of peptides that exert blocking RANKL activity, without TRAIL binding capability. Other peptidomimetics stimulating osteoblatic differentiation could also be similarly envisaged (e.g. towards Bone Morphogenetic Proteins). Thus, these small molecules open new areas of pharmaceutical design for the treatment of bone diseases of inflammatory or non-inflammatory origin.

Inhibition of signalling pathways of bone effectors

The second new pharmaceutical design based on blocking RANKL axis is represented by specific signalling pathway inhibitors. As with the other TNF-receptor family members, the initial step in RANK signalling is the binding to the TNF receptor-associated factor (TRAF) adaptor proteins within the cytoplasmic domain of RANK. The TRAF family includes six members, TRAF-1–6, which have been already shown to bind RANK [1]. The downstream targets of TRAF-6 include: (i) transcription factors such as nuclear factor kappa B (NF-κB), activator protein-1 (AP-1) and nuclear factor of activated T cells (NFAT); (ii) the cascades of mitogen activated protein kinases (MAPK) such as p38 stress kinase, c-Jun N-terminal kinase (JNK), extracellular-signal regulated kinase (ERK); and (iii) the phosphatidyl inositol 3 kinase (PI3K)/AKT (a serine threonine kinase) pathways, which involve the mammalian target of rapamycin (mTOR) [1]. In light of these findings, the NF-κB superfamily plays a central role in osteoclast differentiation and activation as well as in the RANK signalling pathway. In this respect, targeting NF-κB transcription factor has recently emerged as a new therapeutic strategy. Clohisy *et al.* demonstrated that NF-κB signalling blockade abolished implant particle-induced osteoclastogenesis *in vitro* [43]. Similarly, they demonstrated a significant decrease of bone erosion associated with inflammatory arthritis using dominant negative IκB or mutated IκB proteins [44]. In this study, direct administration (1 mg/kg, i.p.) of dominant-negative form of I-κB lacking N-terminal phosphorylation site, significantly blocks NF-κB activation resulting in downregulation of osteoclast recruitment and bone resorption. Synthetic double-stranded oligodeoxynucleotides that act as 'decoy' *cis*-elements that block the binding of nuclear factors to promoter regions of targeted genes, resulting in the inhibition of gene transactivation *in vivo* as well as *in vitro*, have been developed. Thus, decoy oligodeoxynucleotides were demonstrated to be useful in the inhibition of adenocarcinoma cell growth and associated cachexia in mouse model [45]. The effects of decoy DNA/DNA molecules targeting NF-κB on osteoclast differentiation and survival were recently examined [46,47]. These authors revealed that such strategies lead to the induction of osteoclast apoptosis resulting from an inhibition of IL6 expression and upmodulation of caspase 3.

Discussion

Therapeutic strategies that allow the inhibition of bone resorption (via inhibition of osteoclast differentiation or induction of selective apoptosis) are of great importance for the treatment of a large variety of malignant (primitive or metastatic tumor-associated osteolysis) or benign (e.g. aseptic prosthesis loosening and osteoporosis) osteopenic disorders. To date, bisphosphonates represent the standard therapeutic approach for the treatment of osteolytic bone diseases. Although bisphosphonates appear as an effective treatment, undesirable side-effects have been reported, particularly nephrotoxicity and severe hypocalcaemia, when high doses

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are injected intravenously. This has necessitated the development of strategies based on cytokines, peptidomimetics and inhibitors of specific signalling pathways. These new molecules could be used alone or in conjunction with bisphosphonates, thus reducing the amount of bisphosphonate required to provide therapeutic benefit and hence their undesirable effects. New therapeutic approaches of bone erosion have been more recently envisaged, based on the design of small peptidomimetics that are able to block targeted-cytokine biological activities, with particular emphasis on TNFα, RANKL or IL-1 families. The proposed neutralizing strategy is based on the use of peptidomimetics that mimic cytokine receptors such as RANK, 'decoy receptors' such as OPG, or natural antagonists such as IL1-Ra, with the hope that these peptides would exert less pleiotropic effects than the corresponding whole proteins. They represent key molecules to better understand the molecular mechanisms involved in this process and could present various advantages, mainly a reduced immunogenicity, a more targeted effect and multiple applications in which these molecules are implicated (i.e. inhibition of bone resorption and inflammation). The final strategy relates to the blockade of specific signalling pathways currently activated by bone resorption effectors. The use of selective inhibitors of NF-κB blocks osteoclastogenesis and also prevent inflammatory bone destruction *in vivo* [48]. In this context, this therapeutic approach could be very promising for disorders associating bone loss and inflammatory components (e.g. wear debris-induced osteolysis or inflammatory arthritis). In future developments, the high affinity of bisphosphonates to bone could be exploited as a means of delivering novel bone resorption effectors. In a clinically effective treatment, the addition of both components in a complex could lower the quantity of each component required.

In short, the therapeutic strategies based on (i) physiological effector antagonists of bone resorption, (ii) on the development of peptidomimetic of these effectors and (iii) on targeting specific signalling pathways might be evaluated and compared with benefit/risk ratio for the patient. Such approaches could be valuable for osteoarticular diseases such as osteoarthritis, associating several pathological components (i.e. inflammation and bone resorption, cartilage and bone erosion). Additional studies are required to determine the clinical efficacy of these new therapies of bone diseases.

Acknowledgements

This work was supported by INSERM and the Région of Pays de Loire and by a grant from the Loire-Atlantique Committee of the Ligue Contre le Cancer.

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