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Bisphosphonate-Generated ATP-Analogs Inhibit Cell Signaling Pathways

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Abstract

Bisphosphonates are a major class of drugs used to treat osteoporosis, Paget's disease, and cancer. They have been proposed to act by inhibiting one or more targets including protein prenylation, the epidermal growth factor receptor, or the adenine nucleotide translocase. Inhibition of the latter is due to formation in cells of analogs of ATP: the isopentenyl ester of ATP (ApppI) or an AppXp-type analog of ATP, such as AMP-clodronate (AppCCl₂p). We screened both ApppI as well as AppCCl₂p against a panel of 369 kinases finding potent inhibition of some tyrosine kinases by AppCCl₂p, attributable to formation of a strong hydrogen bond between tyrosine and the terminal phosphonate. We then synthesized bisphosphonate preprodrugs that are converted in cells to other ATP-analogs, finding low nM kinase inhibitors that inhibited cell signaling pathways. These results help clarify our understanding of the mechanisms of action of bisphosphonates, potentially opening up new routes to the development of bone resorption, anticancer, and anti-inflammatory drug leads.

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Notes

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Supporting Information

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Figures S1–S18 and Tables S1–S4; quantum chemical calculations; Western blots procedure; preparation and extraction of ApppI and other AMP-metabolites; ApppI quantitation; mitochondrial oxygen consumption assay; synthetic procedures; ¹H and ³¹P NMR; LCMS spectra; references (PDF)

INTRODUCTION

Bisphosphonates are a major class of drugs used to treat bone-related diseases.¹ They are analogs of diphosphate (a. k.a. pyrophosphate), and first-generation “non-nitrogen” bisphosphonates (NNBPs) such as clodronate (**1**, Figure 1), etidronate (**2**), and tiludronate (**3**) were discovered in 1969² as an outgrowth of earlier work³ in which diphosphate was shown to affect hydroxyapatite formation/dissolution. NNBPs were initially thought to have a “physical” mechanism of action, by binding to the bone surface (Figure 2a). In 1992^{4,5} it was then shown that NNBPs were converted to ATP-analogs such as **4** and **5**,^{4,5}(Figure 2b) and it was proposed that these ATP-analogs might inhibit enzymes. Then, “nitrogen-containing” bisphosphonates (NBPs) such as zoledronate (**6**) were developed (Figure 2c) and found to inhibit protein prenylation^{6,7} by inhibiting farnesyl diphosphate synthase (FPPS^{8–13}) which catalyzes the condensation of dimethylallyl diphosphate (DMAPP; **7**) with isopentenyl diphosphate (IPP; **8**) to form farnesyl diphosphate (FPP; **9**), essential for protein prenylation/cell signaling. FPP is also converted by geranylgeranyl diphosphate synthase (GGPPS) to geranylgeranyl diphosphate (GGPP; **10**), again used in protein prenylation. In 2002,¹⁴ **4** was shown to inhibit the mitochondrial ADP/ATP translocase (ANT), providing a basis for the mechanism of action of the NNBPs (Figure 2d). Then in 2009, it was shown that NBPs could generate other ATP-analogs: the isopentenyl ester of ATP, **11**, as well as the dimethylallyl ester, **12**. These form because FPPS inhibition results in large increases in the levels of the FPPS substrates **7** and **8** which are converted to the ATP-esters **11** and **12** (Figure 2e). As with the clodronate analog **4**, compound **11** was shown to inhibit the ANT in mitochondria, leading to cell death.¹⁵

In addition to their role in treating bone-resorption diseases, bisphosphonates kill tumor cells^{16,17} and are being tested in Europe as adjuvant cancer therapeutics.^{18,19} However, their mechanisms of action are still not clear since in recent work^{20,21} it was proposed that NBPs directly inhibit the epidermal growth factor receptor (EGFR), Figure 2f. There was, however, a puzzling “dose discrepancy” between the effects of bisphosphonates in cell-free systems ($IC_{50} \approx 500 \mu M$ in EGFR inhibition assays) versus cell-based assays ($EC_{50} \approx 50 \mu M$), in tumor cell killing,^{20,21} and the $500 \mu M$ value for EGFR inhibition is ~ 5 – 6 orders of magnitude larger than that found with conventional EGFR inhibitors. We thus sought to deduce models for bisphosphonate activity that might help explain these previous observations. We tested three hypotheses: First, NBPs do not directly inhibit the EGFR. Rather, the ATP analogs **11** and **12** are the kinase inhibitors, because these compounds have structural similarity to ATP. Second, with NNBPs such as clodronate, we hypothesized that ATP-analogs such as AppCCl₂p (**4**, adenosine 5'-(beta, gamma-dichloro-methylene) diphosphate) also inhibit kinases. Third, we hypothesized that, if species such as AppCCl₂p inhibited kinases, then lipophilic “pre-prodrug” forms of the NNBPs^{22–24} might act by inhibiting cell signaling pathways. We tested these hypotheses using results for a broad range of NBPs and NNBPs, AppXp ATP-analogs (where X is a variable group that bridges P β and P γ), and AppXp pre-prodrugs in, as appropriate, kinome screens with 369 kinases, 47 individual kinase dose response assays, cell growth inhibition assays using 6 cell lines, Western blots, mitochondrial respiration assays, FPPS and GGPPS inhibition, ApppI formation assays, as well as geranylgeraniol (GGOH) “prenylation rescue” assays.

RESULTS AND DISCUSSION

Bisphosphonates Are not Potent EGFR Inhibitors

We first investigated the effects of 11 bisphosphonates (8 NBPs and 3 NNBP) on EGFR inhibition using a wild type EGFR kinase-domain assay, with poly[Glu:Tyr](4:1) and [γ - ^{33}P]-ATP as substrates. Dose–response curves are shown in Figure S1, and numerical values are summarized in Table 1. The IC_{50} values were many orders of magnitude higher than we find with the known EGFR inhibitors staurosporine ($\text{IC}_{50} = 26 \pm 8 \text{ nM}$; $n = 6$) or erlotinib ($\text{IC}_{50} \approx 380 \pm 250 \text{ pM}$; $n = 2$), ranging from ~ 30 – $40 \mu\text{M}$ (minodronate **13** and tiludronate **3**) to ~ 70 – $100 \mu\text{M}$ (zoledronate **6**, risedronate **14**, and a lipophilic bisphosphonate, **15**),²⁵ to $>100 \mu\text{M}$ for alendronate, *N*-(2-(3 picolyl)aminomethylene) bisphosphonate, ibandronate, pamidronate, etidronate, and clodronate, Table 1, showing that inhibition of the EGFR by many bisphosphonates is very weak. In contrast, we found that the ApppI that accumulates in cells on FPPS inhibition inhibited EGFR kinase activity with an IC_{50} of $9.2 \pm 4 \mu\text{M}$ ($n = 6$). Dose–response curves are shown in Figure S1. So, ApppI is a more potent EGFR inhibitor than any of the bisphosphonates tested or indeed the ATP analog AMP·PNP, which has an IC_{50} of $36 \mu\text{M}$ in this assay. We also prepared and tested the dimethylallyl ester of ATP, ApppD (**12**),^{26,27} finding an $\text{IC}_{50} \approx 14 \mu\text{M}$ (Figure S1).

ApppI has been reported to accumulate in some cells to $>100 \mu\text{M}$ levels, on bisphosphonate treatment.¹⁵ However, given typical ATP concentrations in cells (~ 1 – 5 mM) and known K_{M} [ATP] values ($\sim 5 \mu\text{M}$ ²⁸), it seems that EGFR inhibition by **11** or **12** will not be a major target for cell growth inhibition. In contrast, we found much more potent EGFR inhibition with AppCCl₂p ($\text{IC}_{50} = 640 \pm 120 \text{ nM}$), Figure S1, of interest since AppCCl₂p levels in some cells can reach mM levels.^{15,29} We thus find that bisphosphonates do not directly inhibit the EGFR in the same assays in which we obtain potent, low nM inhibition by the known kinase inhibitors, staurosporine and erlotinib. However, the results do suggest the possibility that some ATP-analogs might potently inhibit this or other kinases, indicating the desirability of a large kinome screen.

Kinome-Screening Results for AppCCl₂p and ApppI

To see whether any kinases were potently inhibited by AppCCl₂p or ApppI, we carried out screens of 369 kinases using the HotSpot assay (Reaction Biology Corporation; 20 mM Hepes, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 0.02% Brij-35, $1 \mu\text{M}$ ATP, single dose duplicate mode at $1 \mu\text{M}$ inhibitor) using both compounds. Results (computed IC_{50} values) are shown in Table 2 for the top 20 hits and complete kinome screen results are in Tables S1 and S2. In order to obtain more accurate results, we then carried out a series of 10-dose, dose–response assays for ApppI (8 kinases; Figure S2) and AppCCl₂p (17 kinases, Figure S3). For the 17 kinases where we obtained both dose–response as well as kinome screen results we found a Pearson *R*-value correlation coefficient $R = 0.96$ between the IC_{50} values for the two data sets (Figure S4).

What the results in Tables 2 and 3 show is that there is potent ($\sim 10 \text{ s nM}$) inhibition of several kinases (e.g., PDGFR α , JAK2, JAK3, and FGFR2) by AppCCl₂p, plus, many other kinases are inhibited at higher concentrations. Moreover, for JAK3 we determined K_{i} values

at 1 and 200 μM ATP levels, and using reported K_M ATP values³⁰ we find $K_i \approx 4\text{--}11$ nM. However, the inhibitory effects of ApppI and ApppD (ApppD dose–response curves for 9 kinases shown in Figures S1 and S5) are very much weaker. These results have several implications. First, since AppCCl₂p levels in clodronate-treated macrophages can reach mM levels²⁹ and ApppI can reach ~ 130 μM ¹⁵ (and ApppD is $\sim 2\times$ higher), numerous kinases will be inhibited although the effects will be much more pronounced with clodronate/AppCCl₂p because the IC₅₀ values there are $\sim 2\text{--}3$ orders of magnitude smaller than with ApppI/ApppD. Second, it has recently been shown³¹ in mice breast cancer models (as well as in one patient) that bisphosphonates bind to granular microcalcifications that are then engulfed by tumor-associated macrophages (TAMs). This is expected to lead to high AppCCl₂p levels in TAMs, and as noted by Junankar et al.,³¹ “TAMs are powerful promoters of tumor growth and meta stasis,³² and extensive macrophage infiltration in many tumor types is associated with poor patient prognosis”.³³ It thus appears that some of the extra-skeletal effects of bisphosphonate therapy especially with breast cancer patients could arise from kinase inhibition in TAMs. In mice treated with NBPs, ApppI levels in osteoclasts reach ~ 20 μM ,³⁴ so direct kinase inhibition is unlikely to be a major contributor to activity. Third, clodronate levels in tissues in patients on systemic clodronate therapy are expected to be low because bisphosphonates bind avidly to bone mineral. However, intriguingly, there are several reports of the anti-inflammatory effects of NNBP, ^{35–42} so our finding that JAK 1/3 and Tyk2 are some of the kinases that are inhibited by AppCCl₂p is of interest since the anti-inflammatory drug Xeljanz functions by inhibiting JAK 1/3.^{43,44} We find a 5 nM IC₅₀ for JAK3 inhibition (and as discussed below, STAT3 involvement in activity with an AppXp analog). Fourth, we see that FGFR2 (the fibroblast growth factor 2 receptor) is inhibited by AppCCl₂p (IC₅₀ ≈ 22 nM), and in earlier work⁴⁵ it was shown that clodronate reduced FGF2-driven cell growth in HUVECs (human umbilical vein endothelial cells), but there was no effect in the absence of FGF2. In that work it was proposed that clodronate bound directly to FGF2, inhibiting its activity, but here, we now have evidence for low nM FGFR2 inhibition by AppCCl₂p, suggesting a direct effect of the clodronate metabolite on FGFR2 cell signaling. Overall then, the kinase inhibition results reveal that there are multiple kinases that are quite potently inhibited (IC₅₀ ≈ 10 nM) by AppCCl₂p, offering new insights into the mechanisms of action of the clodronate.

AppCCl₂p and ApppI Target Different Kinase Classes

When all of the kinase inhibition results are examined, it is apparent that there are four trends that relate to tyrosine versus serine/threonine kinase inhibition by AppCCl₂p and ApppI and kinase inhibition in general. First, the targets for AppCCl₂p and ApppI are different. For the 20 most potent “hits” in the kinome screens, AppCCl₂p targets $\sim 78\%$ tyrosine kinases, highlighted in pink in Table 2, but only 22% of the top 20 are serine/threonine kinases, highlighted in cyan in Table 2. Second, only $\sim 14\%$ of the top 20 hits in the ApppI kinome screen are tyrosine kinases but $\sim 86\%$ of the top 20 are serine/threonine kinases, Table 2. Third, the activities of AppCCl₂p and ApppI when mapped onto kinome dendrograms (using the Kinmap program;⁴⁶ Figure 3a,b) clearly show that the vast majority of AppCCl₂p targets are in the TK (tyrosine kinase) group, while ApppI inhibition is much more widespread and, as noted, favors Ser/Thr kinases. Fourth, AppCCl₂p is a much more

potent kinase inhibitor than is ApppI (or ApppD). What, then, might be the molecular basis for the differential effects (on both activity and selectivity) of AppCCl₂p and ApppI?

Molecular Basis for AppCCl₂p Activity and Selectivity

The more potent and selective activity of AppCCl₂p for Tyr versus Ser/Thr kinases could be due to stronger binding to Tyr kinases in general, model A, or to stronger binding to Tyr versus Ser/Thr OH groups in substrates, model B. The latter model is attractive since the phosphonate group in AppCCl₂p would be expected to form a much stronger hydrogen bond with the aromatic Tyr than with the aliphatic Ser/Thr. This is because, as shown in Figure 4a, there can be strong hydrogen bonding between phosphonate/phenol and phosphonic acid/phenolate species that cannot occur with Ser/Thr due to the very high pK_a values (~16) of aliphatic alcohols. These sorts of hydrogen bonds are well-known in, e.g., salicylates in which the carboxylate interacts with a phenolic OH.⁴⁷

We thus next used a LanthaScreen Eu kinase binding assay with activated JAK3 to determine (in the absence of substrate) the K_i for AppCCl₂p binding. We found $K_i = 73$ nM (Figure S6) which is much higher than the $K_i \approx 4\text{--}11$ nM found in the activity assays (at 1 and 200 μ M ATP). This means that the presence of a substrate Tyr is required for high affinity binding, supporting model B. Then, to test if phosphonate-tyrosine hydrogen bond interactions are indeed significantly different to phosphonate-serine/threonine interactions, we used density functional theory (DFT) and atoms-in-molecules (AIM) theory.⁴⁸

AIM theory is a quantum chemical method that we and others have used to investigate the details of hydrogen bond interactions.⁴⁹ According to AIM theory, every chemical bond has a bond critical point (BCP) at which the first derivative of the charge density, $\rho(\mathbf{r})$, is zero. The $\rho(\mathbf{r})$ topology is described by a real, symmetric, second-rank Hessian-of- $\rho(\mathbf{r})$ tensor, and the tensor trace is related to the bond interaction energy by a local expression of the virial theorem:

$$\text{Tr}(\text{Hessian}) = \nabla^2 \rho(\mathbf{r}) = [2G(\mathbf{r}) + V(\mathbf{r})](4m/\hbar^2) \quad (1)$$

where $\nabla^2 \rho(\mathbf{r})$ is the Laplacian of $\rho(\mathbf{r})$ and $G(\mathbf{r})$ and $V(\mathbf{r})$ are electronic kinetic and electronic potential energy densities, respectively. Negative and positive $\nabla^2 \rho(\mathbf{r})$ values are associated with shared-electron (covalent) interactions and closed-shell (electrostatic) interactions, respectively. In the latter case, one can further evaluate the total energy density, $H(\mathbf{r})$, at the bond critical point:

$$H(\mathbf{r}) = G(\mathbf{r}) + V(\mathbf{r}) \quad (2)$$

A negative $H(\mathbf{r})$ is termed partial covalence, while a positive $H(\mathbf{r})$ indicates a purely closed-shell, electrostatic interaction.⁵⁰ We first used the models shown in Figure 4b to examine the differences in the interactions between a bisphosphonate (modeled here as $\text{Me}(\text{HPO}_3)^-$) and Tyr (modeled as PhOH), versus with Ser/Thr (modeled as MeOH). The optimized geometries for PhOH· $\text{Me}(\text{HPO}_3)$ and MeOH· $\text{Me}(\text{HPO}_3)^-$ are quite similar (Figure 4b and

Table S3.). As shown in Table S3, the hydrogen bond in the Tyr (phenol) model system is clearly stronger, as indicated by a 0.115 Å shorter O'...H distance and a 0.096 Å shorter O'...O distance. AIM-calculated BCP properties show that the OH bonds in PhOH and MeOH are of a covalent nature, having large negative Laplacians. However, the O'...H hydrogen bonds in both systems are by definition of a closed-shell (electrostatic) nature, since they have positive Laplacians. They also exhibit what is called partial covalence, due to a negative $H(\mathbf{r})$, which is 230% larger in the Tyr than in the Ser/Thr model system. The charge densities $\rho(\mathbf{r})$ and electronic kinetic and potential energy densities $G(\mathbf{r})$ and $V(\mathbf{r})$ are also larger (30–45%) in the hydrogenbond in the Tyr versus Ser/Thr model system, meaning a stronger H-bond with the Tyr model. To help confirm this, we carried out two additional sets of calculations, using a difluoromethylene phosphonate $\text{CHF}_2(\text{HPO}_3)^-$ to model AppCCl₂p and $[(\text{MeO})_2\text{PO}_2]^-$ to model ApppI. The optimized structures are shown in Figure 4c,d. All computed properties were very similar to those found with the initial models (Table S3), confirming that the phosphonate hydrogen bond in the Tyr model is much stronger than in the Ser/Thr model. An atomic charge analysis shows that the charge on PhOH in the complexes is ~65% more negative than that for MeOH, due to the stronger ability of the phenyl ring to delocalize charge from the $\text{Me}(\text{HPO}_3)^-$ or $\text{CHF}_2(\text{HPO}_3)^-$ species, providing in essence, additional resonance stabilization that again contributes to a stronger interaction with Tyr.

Another way to view these interactions is to consider the ^1H NMR chemical shifts of the protons involved in hydrogen bonding (Table S3), as we have reported previously,^{47,49} in which partial covalence occurs when ^1H NMR shifts in hydrogen bonded systems reach the 12–14 ppm range (downfield from TMS), while fully shared-electron (covalent) interactions have shifts of ~21 ppm.⁴⁹ With the Tyr models, the computed chemical shifts (~12–13 ppm) are much larger than those of the proton involved in the hydrogen bond in the Ser/Thr model (~6–7 ppm), consistent with the onset of partial covalence, and these ~12–13 ppm shifts are very similar to those we have found in intramolecular hydrogen bonds in salicylates.⁴⁷ So, the Tyr versus Ser/Thr selectivity effects seen with AppCCl₂p are consistent with stronger hydrogen bonding to Tyr, which we compute to be $E \approx 3.9 \text{ kcal mol}^{-1}$ larger than in the Ser/Thr model. A frequency analysis was then used to verify the nature of the stationary points on the respective potential energy surfaces, yielding a G (at 300 K, 1 atm) of 2.85 kcal mol⁻¹, corresponding to a ~100× increase in binding to Tyr. With ApppI, kinase inhibition is very much weaker, likely due to steric repulsions of the bulky isopentenyl group with Tyr or Ser/Thr, an effect that would of course be largest with the more bulky Tyr.

Synthesis of Novel AppXp Analogs and Kinase Inhibition

Given that there are several kinases that are inhibited by AppCCl₂p, the question arises, can more potent species be developed? In the case of bone resorption, the NBP zoledronate is a very effective drug, but there are numerous cases of bisphosphonate-related osteonecrosis of the jaw, and it has been suggested that new NNBP with anti-inflammatory activity might help decrease this risk.⁴⁰ The anti-inflammatory activity of NNBP is also of interest given their potent JAK2 and JAK3 inhibition, plus tiludronate has been shown to reduce cytokine production from activated macrophages,³⁵ to alleviate cachexia in nude mice xenograft models,³⁸ and is reported to be effective in treating erosive osteoarthritis of the hand⁴¹ as

well as knee osteoarthritis³⁹ effects that might all be improved with more active/bioavailable species. Plus, in the case of anticancer activity, kinases might also be targets for NNBP prepro-drugs^{22–24} that form AppXp inhibitors. For example, we find that the activity of AppCCl₂p against wild type EGFR is IC₅₀ ≈ 250 nM, but against five mutants, we find d746–750, 4.3 μM; G719S, 760 nM; L858R, 490 nM; T790M, 270 nM; L858R, T790M, 80 nM, Table 3 and Figure S4.

Since AppCCl₂p is a relatively potent inhibitor of the L858R, T790M double mutant and since this an important drug target, we elected to synthesize a series of AppCCl₂p analogs and test them against the EGFR, as well as against the L858R, T790M mutant. We synthesized eight analogs (**16–23**; Figure 5) containing small substituents at the carbon bridging Pβ and Pγ, as well as four analogs (**5** and **24–26**) containing larger, aryl substituents. The dibromobenzyl species **25** was of interest since the parent bisphosphonate esters were previously shown²² to have potent activity against three human tumor cell lines. As with tiludronate, the “pro tected” dibromobenzyl ester bisphosphonate was converted in cells to the AppXp analog **25**, as shown in the MS and LC-MS results in Figures S7a and S7b. All EGFR inhibition dose–response curves are in Figure S8a,b, and IC₅₀ values are in Table S4. The most potent EGFR inhibitors have CFCl, CCl₂, CBr₂, or C(Me)N₃ bridging groups between Pβ and Pγ, and there is a parabolic dependence of activity on the van der Waal’s surface area of the molecules (Figure S9a). There is also a strong correlation between the pIC₅₀ values for EGFR versus EGFR (L858R, T790M) inhibition for *n* = 13 compounds, with an *R* = 0.96 (Figure S9b), the most active compounds (**23**) being the dibromo-analog of clodronate and (**21**) the methyl-azide, which inhibit the double mutant with IC₅₀ values of 16 and 4 nM, respectively.

The results shown in Table S4 and Figure S9a indicate that there are several contributors to EGFR inhibition by these AppXp species. The most potent inhibitors have highly electron-withdrawing substituents on the bridging carbon. This results in a p*K*_a4 of 7.0 for AppCCl₂p,⁵¹ essentially the same as the 7.1 found with ATP,⁵¹ while the p*K*_a4 of the CH₂ analog **16** is 8.4, meaning qualitatively that protein electrostatic interactions with AppCCl₂p will mimic those found with the ATP substrate. Plus, the phosphonate (O[−])··HO (Tyr) interaction will be favored (at least, at pH ~7). Why then are some AppXp inhibitors better than others? What the parabolic dependence of IC₅₀ on van der Waals’ surface area shows is that activity initially increases with the size of the bridging carbon substituents, with the CMeN₃ and CBr₂ species having most activity, presumably due to increased hydrophobic interactions. However, on addition of a very large (aryl) substituent, activity decreases, due to the onset of steric repulsions with the protein in addition to changes in the p*K*_a4 values and decreased electrostatic/hydrogen bonding interactions. Consistent with the EGFR inhibition results, we find low activity with both the tiludronate and dibromobenzyl analogs (**5** and **25**) in other kinase assays (Figures S10 and S11). The questions then arise: what activities do the parent bisphosphonate pro-drugs, as well as preprodrug analogs, have in cells?

Cell and Organelle Activity of NNBP, AppXps, and AppXp Preprodrugs

We next sought to see how the clodronate-analog bisphosphonates and their preprodrug forms **27–33** (Figure S12) inhibited tumor cell growth, as well as how the corresponding AppXp analogues inhibited the ANT (as measured via mitochondrial respiration). The smaller bisphosphonates had very poor cell (H460) growth inhibition activity, with IC₅₀ values of ~500 μ M. This improved with the addition of an aromatic side-chain, the dibromobenzyl bisphosphonate having an IC₅₀ $\approx$ 26 μ M. However, major increases in activity were only found with the preprodrug species (**27–33**), as shown in Figure S12, with the pivaloyloxy-methylene (Piv) esters and the phosphoramidate analogs of clodronate^{23,24} all having IC₅₀ values in the 0.4–6 μ M range. However, how do the protected NNBP, that is, their AppXp products, actually kill cells?

It has been generally thought that the AppXp analogs of the NNBP target the ANT, the adenine-nucleotide translocase, with Lehenkari et al.¹⁴ reporting that the ANT was inhibited by AppCCl₂p with an IC₅₀ of ~50 μ M and that mitochondrial respiration was likewise inhibited. We thus tested each of the 13 AppXp analogs as well as ApppI in a heart mitochondrial O₂-consumption assay: results are shown in Figure S13. The range in IC₅₀ values was small 0.76–6.4 μ M (Figure S13), and since concentrations of AppCCl₂p in treated cells are typically in the 100s μ M range, then ANT inhibition of mitochondrial respiration is clearly one target for all of the NNBP preprodrugs. However, in some cases, effects on respiration may be minor when compared with effects on kinase inhibition.

Western Blot Evidence for Effects on Cell Signaling Pathways

We next sought to determine whether the Piv-ester of the dibromo analog of clodronate, **31**, had any effects on cell signaling pathways since **31** was a potent NNBP preprodrug and its product **23** (Scheme 1 and Figure S14) was a 100 nM EGFR inhibitor (Table S4). We began by screening for effects on cell signaling pathways in MCF-7 cells because MCF-7 is a high producer of AppCCl₂p from clodronate. Since it seemed possible that multiple kinases and pathways might be targeted, we elected to first investigate the downstream proteins mTOR and p70S6K, chosen because of their central role in many signaling pathways, including osteoclast survival,⁵² using Western blots. As can be seen in Figure 6a–c, the Western blot results in MCF-7 cells show inhibition (IC₅₀ $\approx$ 6–13 μ M) of mTOR and p70S6K phosphorylation, so the NNBP preprodrug **31** (active metabolite AppCBr₂p, **23**) does affect signaling pathways in MCF-7 cells (which have a low EGFR copy number).

Next, since our initial idea was that the EGFR might be targeted by AppXp compounds, we investigated the EGFR pathway in A431 cells, chosen because EGFR is highly expressed. There was little effect of **31** on the autophosphorylation of EGFR, so we hypothesized that there might be involvement of other kinases (that we previously found were potentially inhibited by AppCCl₂p in vitro) and chose to investigate the phosphorylation of STAT3 (signal transducer and activator of transcription 3; Figure S15). STAT3 is involved in cell growth and apoptosis and is phosphorylated by Janus kinases (JAK), and as noted above, we found potent inhibition of JAK1, JAK2, and JAK3 by AppCCl₂p (Table 2). As can be seen in Figure S15, there is again a dose-dependent inhibition (IC₅₀ $\approx$ 7 μ M) of STAT3 phosphorylation by **31**, as found also with clodronate itself in macrophages albeit at a higher

inhibitor concentration.⁵³ So, the NNBP preprodrug **31** (and likely the other analogs) can affect both upstream (STAT3) as well as downstream (mTOR/p70S6K) signaling pathways, consistent with inhibition of one or more kinases.

Cell and Organelle Activity of NBPs: How Do They Work?

The “standard model” for cell growth inhibition by NBPs has been that FPPS and/or GGPPS inhibition⁵⁴ leads to inhibition of protein prenylation, with ApppI formation and ANT inhibition also being of importance. More recently, NBPs have been reported to inhibit the EGFR, but there was a dose discrepancy between cell growth and kinase inhibition. To further probe the NBP mechanism of action, we investigated cell growth inhibition by the 8 NBPs listed in Table 1, compounds that inhibit GGPPS and/or FPPS, in a high-EGFR expressing cell line, MDA-MB-231. We measured growth inhibition activity ($pEC_{50} = -\log_{10} EC_{50}[\mu M]$; dose response curves in Figure S16), then correlated these effects with EGFR (Table 1), FPPS,⁵⁴ and GGPPS⁵⁵ inhibition; GGOH growth inhibition rescue (Figure S16); ApppI accumulation (Figure S17), and with clogP, a measure of lipophilicity. Results are summarized in the Pearson *R*-value correlation matrix/heat map⁵⁴ shown in Figure 7a. There was essentially no correlation ($R \approx 0.09$) between NBP-induced cell growth inhibition and EGFR inhibition. There was, however, a significant correlation between cell growth inhibition and ApppI levels, $R = 0.81$, as well as with GGPPS inhibition, $R = 0.75$, the GGPPS correlation being almost identical ($R = 0.8$) to that we reported previously with a different set of bisphosphonates in a low EGFR expressing (MCF-7) cell line.⁵⁴ The observation that cell growth inhibition correlates with ApppI levels is in accord with the work of Mitrofan et al.¹⁷ who reported that zoledronate-induced apoptosis correlated with high ApppI levels in three cell lines (MCF-7, MDA-MB-436, and RPMI 8226). The correlation with GGPPS inhibition supports a prenylation mechanism, while the ApppI correlation indicates an ANT mechanism, given that kinase inhibition is so weak. That is, there is strong evidence for multiple targeting. While it may seem surprising that we find a much better correlation between cell growth inhibition with GGPPS inhibition as opposed to FPPS inhibition this is precisely what we found previously with MCF-7 cells and a separate set of bisphosphonates,⁵⁶ with better cell activity being found with the more lipophilic NBPs such as **15** that have activity against both FPPS and GGPPS, as well as good cell penetration.

The observation that there are geranylgeraniol (GGOH) growth inhibition rescues with most NBPs has been taken to support a prenylation mechanism. Surprisingly though, Mitrofan et al.¹⁷ noted that in some cells treated with zoledronate + GGOH (GGOH is converted in cells to GGPP) there was essentially no IPP/ApppI production, a remarkable observation. They suggested that this might be due to negative-feedback regulating HMGC_oA reductase activity,^{56,57} but an alternative or additional explanation for the lack of IPP/ApppI accumulation on zoledronate + GGOH treatment is that GGPP in addition to rescuing prenylation-inhibition is a potent (~60 nM) inhibitor of mevalonate kinase⁵⁸ whose inhibition will also block formation of isoprenoids. So, inhibiting mevalonate kinase and/or HMGC_oAR activity will inhibit formation of ApppI and ApppD, meaning that GGOH (or GGPP) can rescue cells from NBP growth inhibition in two ways: first, by providing GGPP for protein prenylation and, second, by blocking formation of ApppI/ApppD.

The correlation between cell growth inhibition and GGPPS inhibition⁵⁴ indicates that there is a key role for protein geranylgeranylation in cell growth inhibition, as found previously with other cell lines,^{59–62} as does the observation that cell growth inhibition is rescued by addition of GGOH (Figure 7). Moreover, these “rescue” effects are dependent on the number of EGFR copies per cell (Figure 7b). In these experiments, we used MCF-7, SW620, SW480, H460, A549, and MDA-MB-231 cell lines which have increasing EGFR copy numbers per cell.^{63–66} We find a correlation between GGOH-rescue and EGFR copy number, most likely though an “indirect effect” due to large numbers of EGFR needing to interact with large numbers of prenylated Ras proteins to function as opposed to any direct EGFR interaction with the bisphosphonates or ApppI/ApppD. Representative correlations between the EGFR copy number and the increase in growth inhibition IC₅₀ on GGOH addition for ibandronate, risedronate, and pamidronate are shown in Figures 7b and S18. Only very small rescue effects are found with pamidronate, consistent with earlier work⁶⁷ suggesting primary targets other than prenylation, for this bisphosphonate.

Taken together, the results discussed above indicate the following: First, NBPs do not significantly inhibit the EGFR. Second, there are no EGFR bisphosphonate-inhibition correlations with cell activity. Third, some kinases are inhibited by ApppI and ApppD, and cell growth inhibition correlates with ApppI levels. However, kinase inhibition is weak, so the ANT is a likely target. Fourth, the correlation between cell growth inhibition and GGPPS inhibition reflects the importance of protein geranylgeranylation. Fifth, there are large increases in GGOH-rescue seen in high EGFR copy number cells, but since EGFR inhibition is so weak, this represents an indirect effect, due to the importance of protein prenylation and FPPS/GGPPS inhibition. Sixth, GGOH-rescues can have additional mechanisms of action beyond prenylation-rescue since the GGPP that is formed in cells from GGOH is a known inhibitor of mevalonate kinase, whose inhibition (together with HMGCoAR degradation) will lead to reduced ApppI/ApppD levels. In brief then, all of our results support the canonical prenylation + ANT mechanism of action for the NBPs and not the kinase-inhibition mechanism, although, for the NNBPs, our results do support an ANT + kinase mechanism. There is, therefore, multitargeting for both NBPs as well as NNBPs. Interestingly, “ApXpp” compounds (in which the $P\alpha$ -O- $P\beta$ bridging oxygen is replaced by, e.g., CHF) have been reported to act as substrates for kinases such as EGFR and SRC,⁶⁸ the terminal $P\beta$ -O- $P\gamma$ group still being capable of substrate phosphorylation. With X = CHF, there is a remarkable ~1000× difference in activity between the R and S epimers, although activity was less than that found with ATP itself.⁶⁸ In the AppXp systems, the terminal $P\beta$ -X- $P\gamma$ group is inactive, however, due to the presence of two P-C bonds, so these compounds can act as kinase inhibitors.

CONCLUSIONS

The results that we have presented above lead to some new views as to the mechanism of action of bisphosphonates. Historically, nitrogen-containing bisphosphonates (such as alendronate, ibandronate, risedronate, and zoledronate) have all been thought to inhibit protein prenylation, and via ApppI, the adenine nucleotide translocase with NNBPs (like clodronate or etidronate) producing other ATP-analogs, AppXp, that likewise target the ANT. More recently, however, a new mechanism of action of NBPs such as risedronate and

zoledronate was proposed to involve direct inhibition of a kinase, the epidermal growth factor receptor or EGFR, but there were puzzling dose discrepancies between cell (~50 μM) and EGFR (~500 μM) inhibition by NBPs. We thus tested a broad range of NBPs against an expressed EGFR kinase domain that was potentially inhibited by known kinase inhibitors finding very weak activity. We then hypothesized that, since it bears strong structural similarity to ATP, the ApppI (and ApppD) that forms from the IPP/DMAPP which accumulates on FPPS inhibition might actually be targeting the EGFR. There was increased activity, and this may contribute in some cases to NBP activity, albeit in a minor way. Remarkably, however, we then discovered that the ATP analogs that form with NNBPs, such as clodronate, were far more potent EGFR inhibitors than was ApppI, as well as potent inhibitors of other cell signaling kinases. Clodronate and its prodrug forms, for example, are converted in cells to the ATP analog AppCCl₂p that we find to be a low nM inhibitor of kinases such as PDGFR α , JAKs, and FGFR2, in addition to being an ANT inhibitor. Our 369-kinase kinome screen and dose–response results indicate that JAK3 is the kinase that is most potently inhibited by AppCCl₂p, of interest since JAK3 is the target for tofacitinib, a potent anti-inflammatory drug, and as discussed above, some NNBPs have anti-inflammatory activity. The JAK/STAT/Tyk2 pathways are targeted by cytokines and are involved in, e.g., rheumatoid arthritis, and of interest here, JAK3 and Tyk2 are inhibited by AppCCl₂p (at 5 and ~230 nM, respectively), as is STAT3 phosphorylation (by **31**), suggesting a possible mechanism for clodronate (or its analogs) in blocking inflammation by targeting the JAK/STAT pathway. Our results also provide evidence that AppCCl₂p targets tyrosine kinases (~78%) in preference to serine/threonine kinases, and the results of DFT and AIM theoretical calculations indicate stronger (~100 $\times$) hydrogen-bond interactions between AppCCl₂p and Tyr versus with Ser/Thr. Finally, the observation that AppXp species are kinase inhibitors opens up new possibilities for the development of “preprodrugs” using the protecting groups (phosphor-amidates, pivaloyloxymethylenes, and isopropoxils) used in antivirals to deliver otherwise highly polar species into cells, to be converted into novel, multitarget inhibitors.

EXPERIMENTAL SECTION

General information, experimental procedures, and characterization data are provided in the Supporting Information.

Kinase Inhibition Assays

All kinase inhibition assays were carried out by the Reaction Biology Corporation (Malvern, PA) using their Kinase HotSpot Profiling system. The base reaction buffers were typically 20 mM Hepes (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 0.02% Brij35, 0.02 mg/mL BSA, 0.1 mM Na₃VO₄, 2 mM DTT, 1% DMSO, and 1 μM ATP. IC₅₀ values were extracted from 3 $\times$ dilution 10-dose dose–response curves.

Cell Growth Inhibition by Bisphosphonates

We used the methods described previously^{13a,14,43} to determine cell growth inhibition by bisphosphonates, as well as cell growth inhibition rescues by GGOH. Cell line authentication was carried out in the Biopolymer/Genomics Core Facility in the School of

Medicine of the University of Maryland via short tandem repeat (STR) analysis using a Promega Geneprint 10 System.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Russell RGG, Bone. 2011; 49(1):2–19. [PubMed: 21555003]
2. Fleisch H, Russell RG, Simpson B, Muhlbauer RC. Nature. 1969; 223(5202):211–212. [PubMed: 5791741]
3. Fleisch H, Russell RG, Straumann F. Nature. 1966; 212(5065):901–903. [PubMed: 4306793]
4. Rogers MJ, Russell RGG, Blackburn GM, Williamson MP, Watts DJ. Biochem Biophys Res Commun. 1992; 189(1):414–423. [PubMed: 1333194]
5. Pelorgeas S, Martin JB, Satre M. Biochem Pharmacol. 1992; 44(11):2157–2163. [PubMed: 1472080]
6. Luckman SP, Coxon FP, Ebetino FH, Russell RG, Rogers MJ. J Bone Miner Res. 1998; 13(11):1668–1678. [PubMed: 9797474]
7. Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G, Rogers MJ. J Bone Miner Res. 1998; 13(4):581–589. [PubMed: 9556058]
8. Cromartie TH, Fisher KJ, Grossman JN. Pestic Biochem Physiol. 1999; 63(2):114–126.
9. van Beek E, Pieterman E, Cohen L, Löwik C, Papapoulos S. Biochem Biophys Res Commun. 1999; 264(1):108–111. [PubMed: 10527849]
10. Martin MB, Arnold W, Heath HT, Urbina JA, Oldfield E. Biochem Biophys Res Commun. 1999; 263(3):754–758. [PubMed: 10512752]
11. Keller RK, Fliesler SJ. Biochem Biophys Res Commun. 1999; 266(2):560–563. [PubMed: 10600541]
12. Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA, Rodan G. Arch Biochem Biophys. 2000; 373(1):231–241. [PubMed: 10620343]
13. Grove JE, Brown RJ, Watts DJ. J Bone Miner Res. 2000; 15(5):971–981. [PubMed: 10804029]
14. Lehenkari PP, Kellinsalmi M, Nääpänkangas JP, Ylitalo KV, Mönkkönen J, Rogers MJ, Azharyev A, Väänänen HK, Hassinen IE. Mol Pharmacol. 2002; 61(5):1255–1262. [PubMed: 11961144]
15. Mönkkönen H, Auriola S, Lehenkari P, Kellinsalmi M, Hassinen IE, Vepsäläinen J, Mönkkönen J. Br J Pharmacol. 2006; 147(4):437–445. [PubMed: 16402039]
16. Mönkkönen H, Kuokkanen J, Holen I, Evans A, Lefley DV, Jauhainen M, Auriola S, Mönkkönen J. Anti-Cancer Drugs. 2008; 19(4):391–399. [PubMed: 18454049]
17. Mitrofan LM, Pelkonen J, Monkkonen J. Bone. 2009; 45(6):1153–1160. [PubMed: 19699819]
18. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Lancet. 2015; 386(10001):1353–1361. [PubMed: 26211824]
19. Hadji P, Coleman RE, Wilson C, Powles TJ, Clézardin P, Aapro M, Costa L, Body JJ, Markopoulos C, Santini D, Di Leo A, Cameron D, Dodwell D, Smith I, Gnani M, Gray R, Harbeck N, Thurlimann B, Untch M, Cortes J, Martin M, Albert US, Conte PF, Ejlertsen B, Bergh J, Kaufmann M, Holen I. Ann Oncol. 2016; 27(3):379–390. [PubMed: 26681681]

20. Yuen T, Stachnik A, Iqbal J, Sgobba M, Gupta Y, Lu P, Colaianni G, Ji Y, Zhu LL, Kim SM, Li J, Liu P, Izadmehr S, Sangodkar J, Bailey J, Latif Y, Mujtaba S, Epstein S, Davies TF, Bian Z, Zallone A, Aggarwal AK, Haider S, New MI, Sun L, Narla G, Zaidi M. *Proc Natl Acad Sci U S A*. 2014; 111(50):17989–17994. [PubMed: 25453081]
21. Stachnik A, Yuen T, Iqbal J, Sgobba M, Gupta Y, Lu P, Colaianni G, Ji Y, Zhu LL, Kim SM, Li J, Liu P, Izadmehr S, Sangodkar J, Scherer T, Mujtaba S, Galsky M, Gomez J, Epstein S, Buettner C, Bian Z, Zallone A, Aggarwal AK, Haider S, New MI, Sun L, Narla G, Zaidi M. *Proc Natl Acad Sci U S A*. 2014; 111(50):17995–8000. [PubMed: 25453078]
22. Zhang Y, Leon A, Song Y, Studer D, Haase C, Koscielski LA, Oldfield E. *J Med Chem*. 2006; 49(19):5804–5814. [PubMed: 16970405]
23. Webster MR, Zhao M, Rudek MA, Hann CL, Freel Meyers CL. *J Med Chem*. 2011; 54(19):6647–6656. [PubMed: 21863853]
24. Webster MR, Kamat C, Connis N, Zhao M, Weeraratna AT, Rudek MA, Hann CL, Freel Meyers CL. *Mol Cancer Ther*. 2014; 13(2):297–306. [PubMed: 24310621]
25. Xia Y, Liu YL, Xie Y, Zhu W, Guerra F, Shen S, Yeddula N, Fischer W, Low W, Zhou X, Zhang Y, Oldfield E, Verma IM. *Sci Transl Med*. 2014; 6(263):263ra161.
26. Turhanen PA. *ACS Omega*. 2017; 2(6):2835–2838.
27. Puljula E, Turhanen PA. *J Chromatogr B: Anal Technol Biomed Life Sci*. 2017; 1063:180–182.
28. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, Meyerson M, Eck MJ. *Proc Natl Acad Sci U S A*. 2008; 105(6):2070–2075. [PubMed: 18227510]
29. Monkkonen H, Rogers MJ, Makkonen N, Niva S, Auriola S, Monkkonen J. *Pharm Res*. 2001; 18(11):1550–1555. [PubMed: 11758762]
30. Wang R, Griffin PR, Small EC, Thompson JE. *Arch Biochem Biophys*. 2003; 410(1):7–15. [PubMed: 12559972]
31. Junankar S, Shay G, Jurczyk J, Ali N, Down J, Pocock N, Parker A, Nguyen A, Sun S, Kashemirov B, McKenna CE, Croucher PI, Swarbrick A, Weilbaecher K, Phan TG, Rogers MJ. *Cancer Discovery*. 2015; 5(1):35–42. [PubMed: 25312016]
32. Joyce JA, Pollard JW. *Nat Rev Cancer*. 2009; 9(4):239–252. [PubMed: 19279573]
33. Bingle L, Brown NJ, Lewis CE. *J Pathol*. 2002; 196(3):254–265. [PubMed: 11857487]
34. Raikonen J, Crockett JC, Rogers MJ, Monkkonen H, Auriola S, Monkkonen J. *Br J Pharmacol*. 2009; 157(3):427–435. [PubMed: 19371349]
35. Monkkonen J, Simila J, Rogers MJ. *Life Sci*. 1998; 62(8):PL95–102. [PubMed: 9489516]
36. Cocco R, Tofi C, Fioravanti A, Nerucci F, Nannipieri F, Zampieri A, Rosini S, Marcolongo R. *Boll Soc Ital Biol Sper*. 1999; 75(11–12):71–76. [PubMed: 11433681]
37. Rovetta G, Monteforte P. *Minerva Med*. 2003; 94(5):353–357. [PubMed: 14973430]
38. Wang CH, Shen YC, Hsieh JJ, Yeh KY, Chang JW. *J Endocrinol*. 2006; 190(2):415–423. [PubMed: 16899574]
39. Rossini M, Viapiana O, Ramonda R, Bianchi G, Olivieri I, Lapadula G, Adami S. *Rheumatology (Oxford, U K)*. 2009; 48(7):773–778.
40. Oizumi T, Yamaguchi K, Funayama H, Kuroishi T, Kawamura H, Sugawara S, Endo Y. *Basic Clin Pharmacol Toxicol*. 2009; 104(5):384–392. [PubMed: 19413658]
41. Saviola G, Abdi-Ali L, Campostrini L, Sacco S, Baiardi P, Manfredi M, Mannoni A, Benucci M. *Mod Rheumatol*. 2012; 22(2):256–263. [PubMed: 21853386]
42. Shima K, Tsuchiya M, Oizumi T, Takano-Yamamoto T, Sugawara S, Endo Y. *Biol Pharm Bull*. 2017; 40(1):25–33. [PubMed: 28049945]
43. Yamaoka K. *Curr Opin Chem Biol*. 2016; 32:29–33. [PubMed: 26994322]
44. Winthrop KL. *Nat Rev Rheumatol*. 2017; 13(4):234–243. [PubMed: 28250461]
45. Rose K, Finger IE, Ferenz KB. *Biomed Pharmacother*. 2011; 65(1):46–51. [PubMed: 21177067]
46. Eid S, Turk S, Volkamer A, Rippmann F, Fulle S. *BMC Bioinf*. 2017; 18(1):16.
47. Li J, Feng X, Zhu W, Oskolkov N, Zhou T, Kim BK, Baig N, McMahon MT, Oldfield E. *Chem - Eur J*. 2016; 22(1):264–271. [PubMed: 26616530]

48. Bader, RFW. Atoms in Molecules: A quantum Theory (International Series of Monographs on Chemistry). 1. Clarendon Press; Oxford: 1994.
49. Arnold WD, Oldfield E. *J Am Chem Soc.* 2000; 122(51):12835–12841.
50. Bader RFW. *J Phys Chem A.* 1998; 102(37):7314–7323.
51. Blackburn GM, Kent DE, Kolkman F. *J Chem Soc, Chem Commun.* 1981; 22:1188–1190.
52. Reuben JS, Dinh L, Lee J, Stateson J, Kamara H, Xiang L, Opperman LA. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2011; 111(2):196–204. [PubMed: 21237434]
53. Glantschnig H, Fisher JE, Wesolowski G, Rodan GA, Reszka AA. *Cell Death Differ.* 2003; 10(10): 1165–1177. [PubMed: 14502240]
54. Zhang Y, Cao R, Yin F, Hudock MP, Guo RT, Krysiak K, Mukherjee S, Gao YG, Robinson H, Song Y, No JH, Bergan K, Leon A, Cass L, Goddard A, Chang TK, Lin FY, Beek EV, Papapoulos S, Wang AHJ, Kubo T, Ochi M, Mukkamala D, Oldfield E. *J Am Chem Soc.* 2009; 131(14):5153–5162. [PubMed: 19309137]
55. Hudock MP, Zhang Y, Guo R-T, Cao R, No JH, Liang P-H, Ko T-P, Chang T-H, Chang S-c, Song Y, Axelson J, Kumar A, Wang AHJ, Oldfield E. *J Med Chem.* 2008; 51(18):5594–5607. [PubMed: 18800762]
56. Garza RM, Tran PN, Hampton RY. *J Biol Chem.* 2009; 284(51):35368–35380. [PubMed: 19776008]
57. Sever N, Song BL, Yabe D, Goldstein JL, Brown MS, DeBose-Boyd RA. *J Biol Chem.* 2003; 278(52):52479–52490. [PubMed: 14563840]
58. Hinson DD, Chambliss KL, Toth MJ, Tanaka RD, Gibson KM. *J Lipid Res.* 1997; 38(11):2216–2223. [PubMed: 9392419]
59. Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, Wesolowski G, Russell RG, Rodan GA, Reszka AA. *Proc Natl Acad Sci U S A.* 1999; 96(1):133–138. [PubMed: 9874784]
60. van beek E, Lowik C, van der Pluijm G, Papapoulos S. *J Bone Miner Res.* 1999; 14(5):722–729. [PubMed: 10320520]
61. Goffinet M, Thoulouzan M, Pradines A, Lajoie-Mazenc I, Weinbaum C, Faye JC, Seronie-Vivien S. *BMC Cancer.* 2006; 6:60. [PubMed: 16539721]
62. Coxon FP, Helfrich MH, Van't Hof R, Sebti S, Ralston SH, Hamilton A, Rogers MJ. *J Bone Miner Res.* 2000; 15(8):1467–1476. [PubMed: 10934645]
63. Zhang GH, Cai LJ, Wang YF, Zhou YH, An YF, Liu YC, Peng Y, Chen ZF, Liang H. *Biochem Pharmacol.* 2013; 86(12):1721–1730. [PubMed: 24161785]
64. Schaeybroeck SV, Kyula J, Kelly DM, Karaïskou-McCaul A, Stokesberry SA, Van Cutsem E, Longley DB, Johnston PG. *Mol Cancer Ther.* 2006; 5(5):1154–1165. [PubMed: 16731747]
65. Zhang XT, Kang LG, Ding L, Vranic S, Gatalica Z, Wang ZY. *Oncogene.* 2011; 30(7):770–780. [PubMed: 20935677]
66. Goetz M, Ziebart A, Foersch S, Vieth M, Waldner MJ, Delaney P, Galle PR, Neurath MF, Kiesslich R. *Gastroenterology.* 2010; 138(2):435–446. [PubMed: 19852961]
67. van Beek ER, Cohen LH, Leroy IM, Ebetino FH, Löwik CWGM, Papapoulos SE. *Bone.* 2003; 33(5):805–811. [PubMed: 14623056]
68. Ni F, Kung A, Duan Y, Shah V, Amador CD, Guo M, Fan X, Chen L, Chen Y, McKenna CE, Zhang C. *J Am Chem Soc.* 2017; 139(23):7701–7704. [PubMed: 28535041]

Figure 1.

Structures of compounds discussed in the text. NBPs are shown in blue.

Figure 2.

Time-line for proposed bisphosphonate mechanisms of action. (a) In 1969, clodronate and etidronate were proposed to affect bone resorption by a "physical" mechanism, involving direct bone-binding, inhibiting hydroxyapatite dissolution. (b) In 1992, clodronate and etidronate were shown to be converted to ATP analogs, AppXp, in the slime mold *Dictyostelium discoideum*, a model for osteoclasts. (c) In 1998–2000, next generation NBPs such as risedronate were shown to block protein prenylation by inhibiting farnesyl diphosphate synthase. (d) In 2002, it was shown that AppCCl₂p inhibited the mitochondrial adenine nucleotide translocase (ANT). (e) In 2009, it was shown that NBPs such as zoledronate led to accumulation of isopentenyl diphosphate and dimethylallyl diphosphate which were then converted to ApppI and ApppD, with ApppI being found to inhibit the ANT. (f) In 2015, it was reported that NBPs such as risedronate and zoledronate could act by directly inhibiting the epidermal growth factor receptor (EGFR).

Figure 3.

Kinase inhibition results for AppCCl₂p (4) and ApppI (11) mapped onto a Kinmap dendrogram showing more potent and selective Tyr-kinase inhibition by AppCCl₂p. (a) Kinmap dendrogram for AppCCl₂p. (b) Kinmap dendrogram for ApppI. The size of the circle was set in Kinmap as $100 + (50 \text{ pIC}_{50} [\mu\text{M}])$ so the larger the circle, the lower the IC₅₀. Red = Tyr kinase; cyan = Ser/Thr kinase. AppCCl₂p inhibits almost exclusively Tyr-kinases but ApppI inhibits a broader range of kinases, albeit only weakly.

Figure 4. Schematic illustration of hydrogen bonding of phenol/phosphonate and geometry-optimized structures. (a) A phenol/phosphonate hydrogen bond. (b) Geometry-optimized structures of phenol or methanol with methyl phosphonate. (c) As in panel b but phenol or methanol with difluoromethyl phosphonate. (d) Phenol or methanol with a dimethyl phosphate. Geometries, bond critical point values, and predicted ¹H NMR shifts are in Table S3.

16, X=CH₂; 17, X=CHF; 18, X=CF₂; 19, X=CFCl; 20, X=CFBr;
21, X=CMeN₃; 22, X=CClBr; 23, X=CBr₂; 24, X=CHOC₆H₄-*p*-Cl;
25, X=CHCH₂C₆H₃-*m,p*-Br₂; 26, X=CHSC₆H₃-*m,p*-Br₂

Figure 5.
Structures of AppXp analogues synthesized.

Figure 6.

Western blot results for effects of **31** on mTOR and p70S6K phosphorylation. The stacked plots are summed photodensitometer traces; representative gels are shown below, and dose-response curves and IC_{50} values (from summed intensities of 3 blots) are shown in the insets. (a) mTOR in MCF7 cells induced with serum. (b) p70S6K in MCF-7 cells induced with serum. (c) p70S6K in MCF-7 cells induced with EGF.

Figure 7.

Correlations between cell growth inhibition and various properties and GGOH-rescue results with three bisphosphonates in different cell lines. (a) Correlation between MD-MBA-231 cell growth inhibition pEC₅₀ ($= -\log_{10} \text{EC}_{50} [\mu\text{M}]$) and GGPPS inhibition pIC₅₀; GGOH rescue factor (the ratio of the EC₅₀ with or without 20 μM GGOH); clogP; log₁₀[Apppl; μM]; and FPPS inhibition pIC₅₀ and EGFR pIC₅₀. The numbers shown in the matrix are the Pearson *R*-value correlation coefficients. Green colors highlight strong correlations, yellow indicates intermediate, and red indicates no significant correlation. (b) GGOH x-fold rescue of the growth inhibition of six cell lines for three bisphosphonates as a function of EGFR copy number. The *R*-values for two bisphosphonates are high but for pamidronate is not significant. The high correlation between cell growth inhibition and GGPPS inhibition (*R* = 0.75) in panel a is very similar to that seen previously with MCF-7 cells and other bisphosphonates (*R* = 0.80). The correlation between cell growth inhibition and ApppI levels is also very high (*R* = 0.81), and taken together these results indicate the importance of

multitargeting with both protein geranylgeranylation as well as ApppI/AppppD inhibitors contributing to bisphosphonate activity.

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Scheme 1.
Conversion of the Preprodrug 31–23

Table 1

IC₅₀ Values for EGFR Inhibition by Bisphosphonates, Phosphonate-Containing ATP Analogs, and Known Inhibitors

compounds	IC ₅₀ (μ M)
erlotinib	$(3.8 \pm 0.25) \times 10^{-4}$ ($n = 2$)
staurosporine	0.026 ± 0.008 ($n = 6$)
AppCCl ₂ P (4)	0.64 ± 0.13 ($n = 3$)
AppPI (11)	9.2 ± 4 ($n = 6$)
AppPD (12)	14
minodronate (13)	30
AMP-PNP	36
tiludronate (3)	43 ± 12 ($n = 2$)
zoledronate (6)	72 ± 12 ($n = 2$)
risedronate (14)	97
BPH-1222 (15)	100
alendronate	220
<i>N</i> -(2-(3-picolyl)AMDP)	300
ibandronate	430
pamidronate	690
etidronate (2)	740

Table 2Top 20 Hits from 369 Kinase Screens with AppCCl₂p (4) and ApppI (11)^a

Kinase (+4)	IC ₅₀ (μM)	Kinase (+11)	IC ₅₀ (μM)
JAK3	1.5E ⁻⁰³	EGFR	1.0
PDGFRa	0.01	MST4	1.5
JAK2	0.02	TYK1/LTK	1.5
FGFR2	0.05	CDK7/cyclin H	1.6
DDR2	0.08	Aurora B	1.8
ABL1	0.09	SGK3/SGKL	2.4
PKCepsilon	0.09	PLK1	2.6
JAK1	0.10	NEK7	2.7
FRK/PTK5	0.11	ERBB4/HER4	2.7
RET	0.12	DLK/MAP3K12	3.4
FLT3	0.12	ULK3	3.4
PKCd	0.14	ASK1/MAP3K5	3.4
LYN B	0.21	ERN1/IRE1	3.5
ABL2/ARG	0.21	NEK1	3.9
EGFR	0.21	PKCtheta	3.9
DYRK3	0.22	P38d/MAPK13	4.0
JNK2	0.23	ROS/ROS1	4.0
TYK2	0.23	STK25/YSK1	4.1
FGFR1	0.24	GSK3b	4.3
c-MET	0.25	YSK4/MAP3K19	4.3

^aPink = Tyr kinase; cyan = Ser/Thr kinase.

Table 3Dose-Response IC₅₀ Values for AppCCl₂p Inhibition of 18 Kinases^a

Kinase	IC ₅₀	Kinase	IC ₅₀
PDGFRα	4 nM	FRK/PTK5	100 nM
JAK3	5 nM	RET	140 nM
JAK2	13 nM	EGFR (T790M)	270 nM
FGFR2	22 nM	EGFR (L858R)	490 nM
PKCε	43 nM	EGFR	560 nM
DDR2	47 nM	EGFR (G719S)	760 nM
JAK1	66 nM	EGFR (d746–750)	4.3 μM
ABL1	79 nM	BRAF	6.4 μM
EGFR (L858R, T790M)	80 nM	BRAF (V599E)	230 μM

^aPink = Tyr kinase; cyan = Ser/Thr kinase.