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Classical Anticytokinins Do Not Interact with Cytokinin Receptors but Inhibit Cyclin-dependent Kinases*[S]

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Cytokinins are a class of plant hormones that regulate the cell cycle and diverse developmental and physiological processes. Several compounds have been identified that antagonize the effects of cytokinins. Based on structural similarities and competitive inhibition, it has been assumed that these anticytokinins act through a common cellular target, namely the cytokinin receptor. Here, we examined directly the possibility that various representative classical anticytokinins inhibit the *Arabidopsis* cytokinin receptors CRE1/AHK4 (cytokinin response 1/*Arabidopsis* histidine kinase 4) and AHK3 (*Arabidopsis* histidine kinase 3). We show that pyrrolo[2,3-*d*]pyrimidine and pyrazolo[4,3-*d*]pyrimidine anticytokinins do not act as competitors of cytokinins at the receptor level. Flow cytometry and microscopic analyses revealed that anticytokinins inhibit the cell cycle and cause disorganization of the microtubular cytoskeleton and apoptosis. This is consistent with the hypothesis that they inhibit regulatory cyclin-dependent kinase (CDK) enzymes. Biochemical studies demonstrated inhibition by selected anticytokinins of both *Arabidopsis* and human CDKs. X-ray determination of the crystal structure of a human CDK2-anticytokinin complex demonstrated that the antagonist occupies the ATP-binding site of CDK2. Finally, treatment of human cancer cell lines with anticytokinins demonstrated their ability to kill human cells with similar effectiveness as known CDK inhibitors.

Cytokinins are plant hormones that play essential roles in the regulation of various aspects of plant growth and development (1). They include a variety of chemicals with different degrees of structural similarity, some of which occur naturally in plants, and others that are known only as synthetic compounds. The natural cytokinins are adenine derivatives that can be classified according to the nature of their N^6-side chain as either isoprenoid (zeatin) or aromatic (benzyladenine) cytokinins.

Cytokinins are key regulators of the plant cell cycle, and the induction of cell division is considered diagnostic for this class of plant hormones. The molecular basis of this activity is only partially understood and may differ in different cell types. Cytokinins have been found to control tyrosine dephosphorylation and activation of $p34^{cdc2}$-like H1 histone kinase (2), as well as the transcriptional activation of cyclin D3 (3). Some of the many physiological and developmental processes that are controlled by cytokinin, such as the formation and activity of shoot apical meristems, floral development, the breaking of bud dormancy, and seed germination (4–8), are at least in part functionally linked to cell cycle control.

Recently, several cytokinin receptors were identified in *Arabidopsis* (9–12) and *Zea mays* (13). To date, three cytokinin receptors have been identified in *Arabidopsis*, AHK2,[4] AHK3, and CRE1/AHK4. All are membrane-located sensor histidine kinases with a predicted extracellular ligand-binding domain and cytoplasmic His kinase and receiver domains. It has been shown that the cytokinin signal is transmitted by a multistep phospho-relay system through a complex form of the two-component signaling pathway that has long been known in prokaryotes and lower eukaryotes. Among higher eukaryotes, the two-component signaling pathway is only found in plants (reviewed by Refs. 14–17).

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[4] The abbreviations used are: AHK2, *Arabidopsis* histidine kinase 2; AHK3, *Arabidopsis* histidine kinase 3; CRE1/AHK4, cytokinin response 1/*Arabidopsis* histidine kinase 4; CDK, cyclin-dependent kinase; tZ, *trans*-zeatin; MI, mitotic index; ANCYT1, 3-methyl-7-pentylaminopyrazolo(4,3-*d*)pyrimidine; ANCYT2, 4-(cyclopentylamino)-2-methylthiopyrrolo(2,3-*d*)pyrimidine; ANCYT3, 4-(cyclobutylamino)-2-methylpyrrolo(2,3-*d*)pyrimidine; HU, hydroxyurea.

The development of agonists and antagonists of a particular physiological effect is useful in mechanism-of-action studies of biologically active natural products. The design of potential cytokinin antagonists has been based on the assumptions that 1) active cytokinins bind to one or more cellular receptor sites and 2) it should be possible to prepare compounds that have minimal cytokinin activity but retain sufficient structural similarity to the cytokinins to permit them to compete for available cytokinin receptor sites, thereby diminishing the biological activity of cytokinins. The potent naturally occurring cytokinin N^6-isopentenyladenine served as the basis for initial structure-activity studies. Modification of the heterocyclic purine system yielded the first analogues with antagonistic activity that greatly reduced cytokinin activity in bioassays (18, 19). Consequently, a number of substituted pyrrolo[2,3-*d*]pyrimidines, pyrazolo[4,3-*d*]pyrimidines, *s*-triazines, *N*-benzyl-*N*′-phenylureas, and *N*-arylcarbamates were subsequently prepared and tested for their ability to inhibit cytokinin-promoted processes in various bioassays, and a number of them were identified as potential anticytokinins (reviewed by 20). Because of their structural similarity to natural cytokinins and because their antagonistic effects were reversible by increasing the cytokinin concentration, it was hypothesized that these compounds work through interaction with a common cellular target, *viz* the cytokinin receptor (20). However, until recently, direct proof that cytokinin receptors are the sites of cytokinin-anticytokinin interactions was lacking because no cytokinin receptors had been identified. Recent advances in our understanding of cytokinin signaling motivated us to re-examine anticytokinin modes of action.

Here we show that representative anticytokinins are not competitive inhibitors of two *Arabidopsis* cytokinin receptors. Furthermore, using mainly the potent anticytokinin 3-methyl-7-pentylaminopyrazolo[4,3-*d*]pyrimidine (ANCYT1) as a representative example, we also show that anticytokinins inhibit cell cycle progression and cause cellular changes consistent with responses to known CDK inhibitors. We demonstrate CDK inhibition by anticytokinins in plants and humans and reveal the binding of ANCYT1 to the ATP-binding pocket of human CDK2. The observed activity of anticytokinins in human cancer cells makes them new candidates for drug research and development.

EXPERIMENTAL PROCEDURES

Chemicals—*trans*-zeatin was obtained from Olchemim Ltd. (Olomouc, Czech Republic). The methods used to synthesize and characterize the anticytokinin analogues were as described previously (21–23). Radiolabeled *trans*-zeatin ([2-^{3}H]zeatin) was obtained from Dr. Jan Hanuš (Isotope Laboratory, Institute of Experimental Botany, AS CR, Prague, Czech Republic).

Bacterial Cytokinin Assay—*Escherichia coli* strains KMI001 harboring the plasmid pIN-III-AHK4 and pSTV28-AHK3, respectively, were described (10, 12). Bacterial cytokinin assays were performed as described in Ref. 24.

Fractionation of E. coli and Binding Assay on Microsomes—CRE1/AHK4- and AHK3-expressing *E. coli* strains (10, 12) were grown to $A_{600} \sim 1$ at 25 °C and then fractionated into periplasmic, cytoplasmic, and membrane fractions. Fractionation and binding assays with *E. coli* membranes were carried out as described previously in Ref. 25.

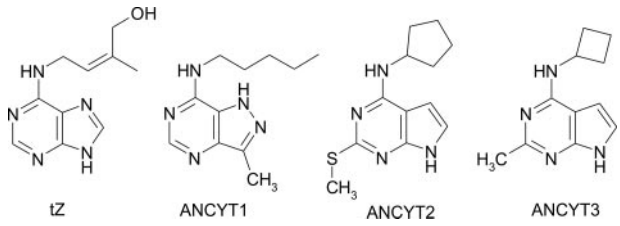

FIGURE 1. **Structure of zeatin and of different anticytokinins used in this study.** tZ, *trans*-zeatin.

Arabidopsis P_{ARR5}*::GUS Reporter Gene Assay*—*Arabidopsis* plants (*Arabidopsis thaliana* (L.) Heynh. accession Col-0) harboring P_{ARR5}*::GUS* gene reporter were described (26). The assay was carried out as described in Ref. 27 with slight modification. Seedlings were grown for 2–3 days (22 °C, 16 h light/8 h dark) in a 6-well plates (TPP, Switzerland), and then cytokinin and test compounds or solvent (Me_2SO, final concentration 0.1%) were added as microaliquots to the desired final concentration. The seedlings were then incubated for 17 h at 22 °C in the dark.

Protein Kinase Assays—The recombinant human protein kinases used for the selectivity screening of anticytokinins (see supplemental Table 1 and Fig. 5*B*) were produced and assayed as described in Refs. 28 and 29. Protein extraction and purification of *Arabidopsis* CDKs by binding to $p13^{suc1}$ beads or immunoprecipitation with antibodies specific to *Arabidopsis* CDKA;1 and CDKB1;1 and protein kinase activity measurements were carried out as described in Refs. 30 and 31, respectively.

Protein X-ray Crystallography—Expression, purification, and crystallization of monomeric human CDK2, as well as ligand introduction, data collection, processing, structure solution, and refinement, were all carried out using methods analogous to those previously described for complex structures with non-cytokinin CDK2 ligands (28, 29). Data collection and refinement statistics for the CDK2-ANCYT1 complex are presented in Table S2 (supplemental Table 2).

Cell Cycle and Apoptosis Study—Root tip meristems of *Vicia faba* were synchronized as described previously in Ref. 32, and the relative DNA contents of *V. faba* nuclei isolated from root tips were analyzed by flow cytometry analysis as described in Ref. 33. The frequencies of prophase and metaphase cells, the mitotic index (MI), were determined in squash preparations and stained according to the standard Feulgen procedure. The percentage of MI was obtained from randomly chosen samples of 1,000–2,000 cells from each treated variants and from the control cells.

Immunofluorescence Staining of Microtubules—Root tips or cultured cells were fixed for 1 h in 3.7% paraformaldehyde and processed for immunofluorescence as described in Ref. 34.

Testing of Cytotoxicity—The human breast carcinoma MCF-7, human chronic myelogenous leukemia K-562, and human osteogenic sarcoma HOS cell lines (obtained from ATCC, Rockville, MD) were used for cytotoxicity determinations of the tested anticytokinins using a calcein AM assay as described in Ref. 35.

RESULTS

Fig. 1 shows the chemical structures of the three anticytokinins ANCYT1, ANCYT2 (4-(cyclopentylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine), and ANCYT3 (4-(cyclobutyl-

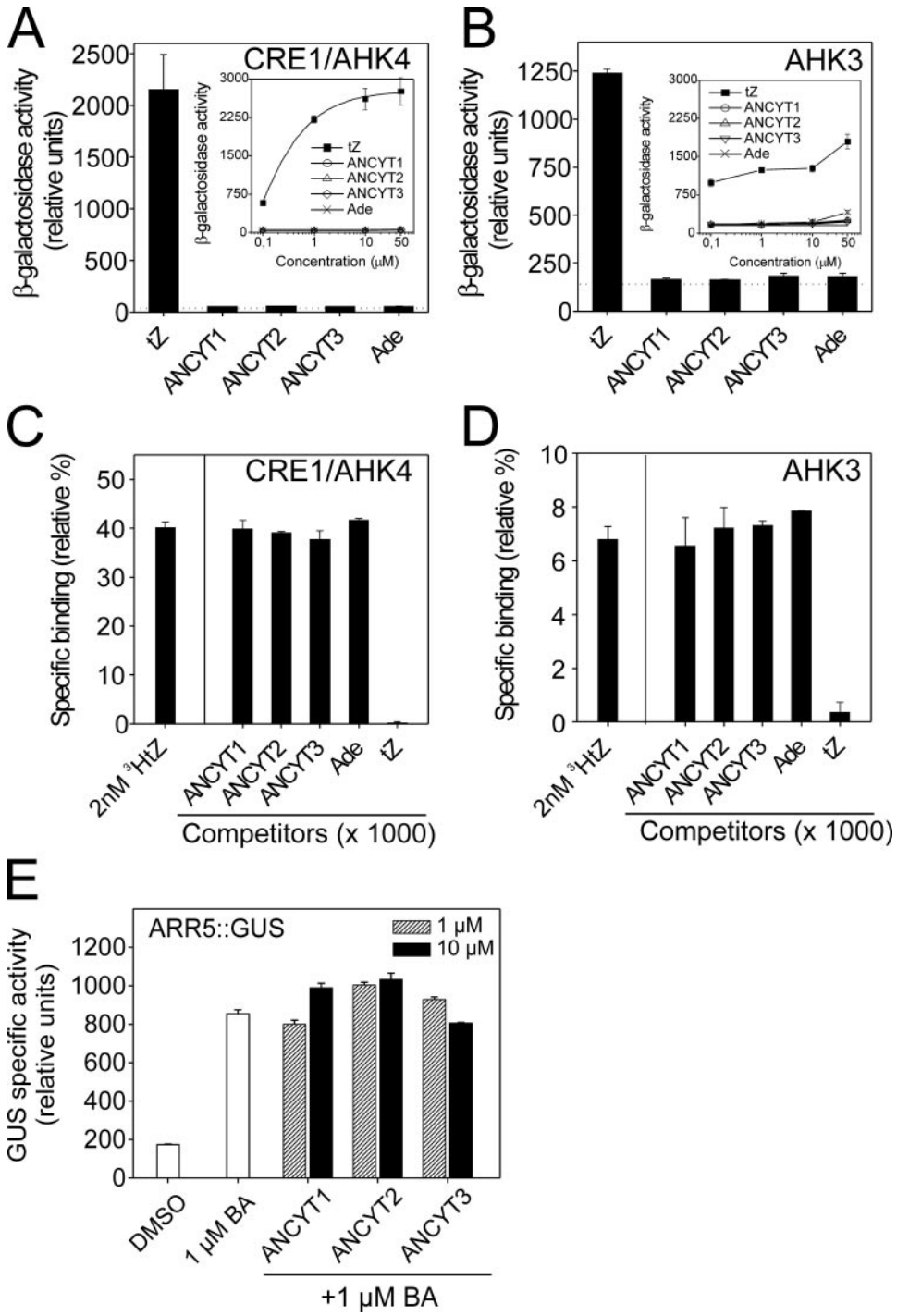

FIGURE 2. **Cytokinin receptor studies.** *A* and *B*, comparison of the sensitivity of CRE1/AHK4 (*A*) and AHK3 (*B*) to 1 μM ANCYT1, ANCYT2, and ANCYT3, adenine (*Ade*, negative control) and *trans*-zeatin (tZ, positive control) in the bacterial assay. The activity of non-induced strains is indicated by the *dotted line*. *Insets* show activation of the cytokinin receptors by the compounds in a dose-dependent manner. *Error bars* show S.D. ($n = 3$). *C* and *D*, competitive binding assay with CRE1/AHK4- (*C*) and AHK3-containing (*D*) *E. coli* membranes. Binding of 2 nM [2-^{3}H]zeatin (*^{3}HtZ*) was assayed together with a 1,000-fold higher concentration of ANCYT1, ANCYT2, and ANCYT3 with adenine as negative control and unlabeled tZ as positive control. *Error bars* show S.D. ($n = 2$). *E*, effect of anticytokinins on induction of the P_{ARR5}::*GUS* gene by cytokinin. P_{ARR5}::*GUS* transgenic *Arabidopsis* seedlings were incubated with 1 μM benzyladenine (*BA*) in the presence or absence of 1 and 10 μM concentration of ANCYT1, ANCYT2, and ANCYT3; Me_2SO (*DMSO*) (0.1%) was tested as solvent control. *Error bars* show S.D. ($n = 3$).

amino)-2-methylpyrrolo[2,3-*d*]pyrimidine), which were selected for this study as being the most active compounds known in their respective substance class (20).

We initially tested the activity of the compounds in the classical tobacco callus growth assay for cytokinins, using an experimental design similar to that described by Hecht (21) and Skoog *et al.* (36). Callus growth increased with increasing cytokinin (*trans*-zeatin) concentration, reaching a maximum at 0.5–1 μM (see supplemental Fig. S1). Growth was inhibited by increasing concentrations of anticytokinin, and ANCYT1 almost completely inhibited callus growth at a concentration of 10 μM (see supplemental Fig. S1).

To investigate whether or not the growth inhibitory effect of anticytokinins results from the blocking of cytokinin receptors, we studied their interactions with the CRE1/AHK4 and AHK3 receptors of *Arabidopsis*. For this, we used *E. coli* reporter strains expressing single cytokinin receptors and the cytokinin-activated reporter gene *cps*::*lacZ* (10, 12, 24). Data presented in Fig. 2, *A* and *B*, show that none of these anticytokinins was able to activate the receptors, even at a concentration 500-fold greater than that required for receptor activation by *trans*-zeatin.

Competitive inhibition of *in vitro* binding of *trans*-zeatin to CRE1/AHK4 and AHK3 by the proposed antagonists was investigated. After fractionation of *E. coli* cells, the presence of the CRE1/AHK4 and AHK3 proteins in isolated membranes, but not in the periplasm and cytoplasm fractions, was verified by equilibrium dialysis using [2-^{3}H]zeatin (not shown). A competitive binding assay of the representative anticytokinins was then carried out, employing unlabeled *trans*-zeatin and adenine as positive and negative controls, respectively. Binding of radioactively labeled *trans*-zeatin to CRE1/AHK4 and AHK3 was inhibited competitively by unlabeled *trans*-zeatin (Fig. 2, *C* and *D*). In contrast, neither adenine nor any of the anticytokinins competed with *trans*-zeatin for receptor binding, even at 1,000-fold excess.

To support our observation that representative anticytokinins do not compete with cytokinin for binding to the cytokinin

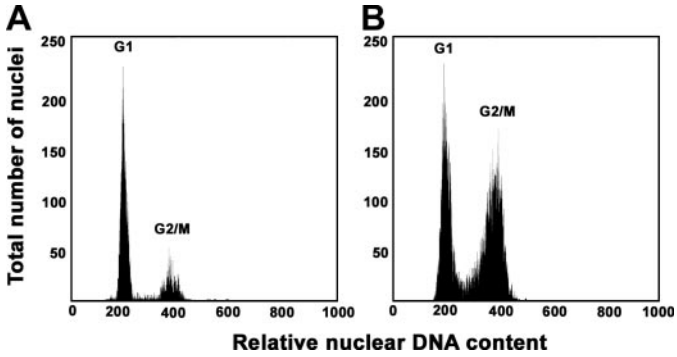

FIGURE 3. **Flow cytometric analysis of nuclear DNA content in control and ANCYT1-treated cells.** *V. faba* root meristem cells were synchronized with HU. ANCYT1 (400 μM) was applied immediately after HU removal, and 10 h later, the DNA content of both control cells (*A*) and ANCYT1-treated cells (*B*) was measured.

receptors, we next determined whether the anticytokinins are able to block cytokinin primary signal transduction. *ARR5* is a member of the type-A response regulators identified as cytokinin primary response genes (26). We used transgenic *Arabidopsis* seedlings harboring the P_{ARR5}::*GUS* reporter (26, 27) to test the effects of ANCYT1, ANCYT2, and ANCYT3 on induction of ARR5 triggered by the cytokinin benzyladenine. Data presented in Fig. 2*E* show that none of the anticytokinins was able to reduce the level of ARR5::GUS.

The activity that is measured by most cytokinin bioassays is the induction of cell division. To explore the activity of anticytokinins on this process, we measured their inhibitory activity on the cell cycle directly, choosing ANCYT1 as a typical example. The effect of ANCYT1 on cell division was studied in *Arabidopsis* cell suspension cultures and *V. faba* root meristems. In several independent experiments, the MI was about 5–7% in the control *Arabidopsis* cells, whereas a significant decrease in MI to 1.5% was observed after treatment with 100 μM ANCYT1. No significant mitotic activity was detected in cells treated with a higher concentration (200 μM) of ANCYT1.

In asynchronous root meristems of *V. faba*, the MI decreased from 8% in the control to 2% after a 12-h treatment with 400 μM ANCYT1. To characterize further the inhibitory effect of ANCYT1 on cell cycling, root meristem cells of *V. faba* were synchronized with hydroxyurea (HU). Synchronization of root meristems increased MI from 8 to 55% as counted 7 h after HU removal in control cells. ANCYT1 was applied immediately after HU removal. Flow cytometric analysis showed that the proportion of cells in G_1 increased 10 h after release from the HU block in the untreated control, indicating that control cells progressed completely through mitosis (Fig. 3*A*); in contrast, a significantly larger proportion of the cells treated with 400 μM ANCYT1 still retained the G_2/M DNA content at this time point (Fig. 3*B*). Together with the observed decrease in MI, these data indicate that ANCYT1 treatment inhibited the G_2/M transition.

Microscopic observation of both *Arabidopsis* and *V. faba* cells revealed that aberrant mitotic chromosome arrangements rather than regular metaphases were frequently present in mitotic cells that had been treated with ANCYT1. Immunofluorescent labeling of tubulin showed that abnormalities of cell cycle-specific arrays of microtubules were induced by

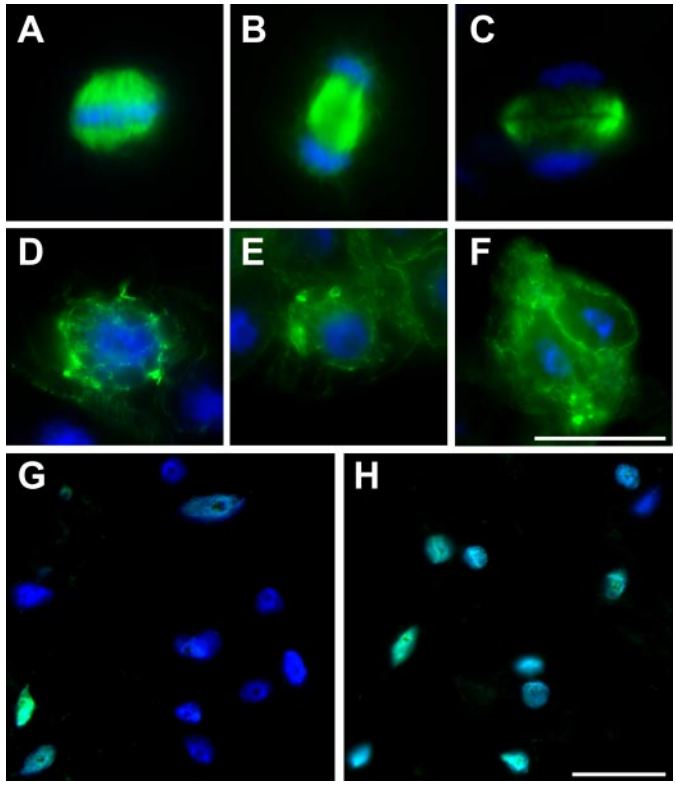

FIGURE 4. **Changes at the cellular level after ANCYT1 treatment.** Immunofluorescent visualization of the microtubular cytoskeleton in *A. thaliana* cells treated with ANCYT1 for 6 h is shown. α-Tubulin was visualized with fluorescein isothiocyanate (*green*) and chromatin with 4′,6-diamidino-2-phenylindole (*blue*). *A–C*, control *Arabidopsis* cells showing a metaphase spindle (*A*), an anaphase spindle (*B*), and a phragmoplast (*C*). *D–F*, *Arabidopsis* cells treated with 100 μM ANCYT1: cell arrested in G_2/M with microtubules randomly arranged around the nucleus (*D*), collapsed interphase microtubules forming circles in cytoplasm (*E*), and late telophase cell with aberrant microtubules (*F*). *Bar* = 10 μm. *G* and *H*, apoptotic changes in ANCYT1-treated cells. Immunofluorescent labeling of double strand DNA breaks in *Arabidopsis* suspension cells is shown. double-stranded DNA breaks were visualized after bromodeoxyuridine incorporation with mouse anti-bromodeoxyuridine antibody and anti-mouse-fluorescein isothiocyanate-conjugated secondary antibody (*green*). Chromatin was visualized with 4′,6-diamidino-2-phenylindole (*blue*). *G*, control *Arabidopsis* cells. *H*, *Arabidopsis* cells treated with 100 μM of ANCYT1 for 3 h.

ANCYT1 in *Arabidopsis* cells after treatment with a dose of 100 μM and were more pronounced at 200 μM (Fig. 4, *A–F*). Normal mitotic microtubule arrays typical of metaphase spindles, anaphase spindles, and cytokinetic apparatus phragmoplasts were observed in control *Arabidopsis* cells (Fig. 4, *A–C*, respectively). In contrast, ANCYT1-treated *Arabidopsis* cultures contained cells in pre-prophase, with microtubules randomly arranged in the nuclei and with highly condensed chromatin and persistent nuclear envelopes. These results are consistent with our observation that ANCYT1 blocked or delayed the G_2/M transition (Fig. 4*D*). ANCYT1 also affected the organization of mitotic microtubules, causing a collapse of the microtubular cytoskeleton, accompanied by a strong affinity of the randomly arranged microtubules for chromatin and by the formation of irregular microtubule arrays, such as circles in the cytoplasm (Fig. 4*E*). The microtubules were also clustered randomly around newly forming daughter nuclei in telophase of ANCYT1-treated cells (Fig. 4*F*). Further significant cellular effects induced by ANCYT1 included apo-

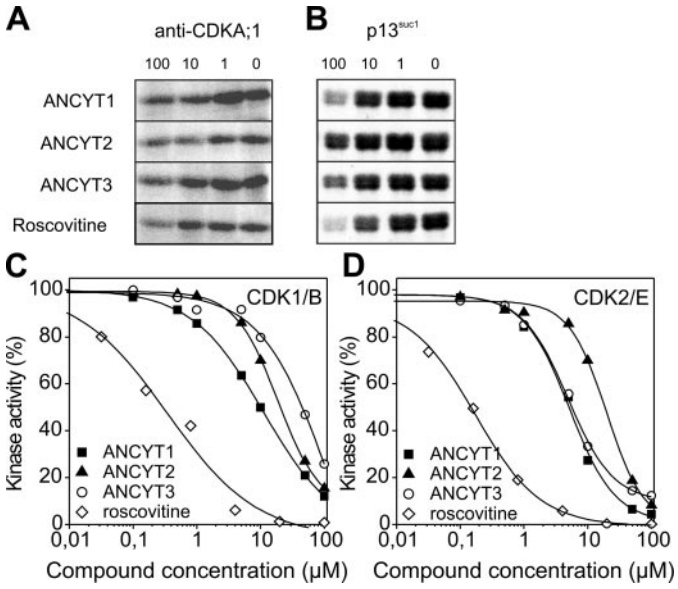

FIGURE 5. **Inhibition of plant and human cyclin-dependent kinases.** *A* and *B*, CDKA;1 kinase was immunoprecipitated with anti-CDKA;1 antibody (*A*) or bound to p13^{suc1}-Sepharose (*B*) from *Arabidopsis* suspension cell extract and assayed in the presence of 0, 1, 10, and 100 μM of the tested compounds. *C* and *D*, inhibition of human recombinant CDK1/cyclin B (*C*) and CDK2/cyclin E (*D*). Activity of CDK toward histone H1 was assayed in the presence of 15 μM ATP and varying concentrations of the tested compounds.

ptotic nuclear changes. Although in the control *Arabidosis* cells, only 1.5–2% of cells showed apoptotic DNA double strand breaks, up to 30% of the cells were apoptotic following a 3-h treatment with 50–100 μM ANCYT1 (Fig. 4, *G* and *H*). Similar changes in microtubule organization and induction of apoptosis were also observed after treatment of *V. faba* root meristems with ANCYT1 (not shown).

The cellular abnormalities observed after anticytokinin treatment are reminiscent of changes caused by roscovitine, a known inhibitor of the pivotal mammalian cell cycle regulator CDK2 (37). We therefore tested the influence of anticytokinins on CDK activity of a key plant cell cycle regulator, CDKA;1. CDKs were purified from an *Arabidopsis* suspension cell culture by two different methods, *viz* by immunoprecipitation with an anti-CDKA;1 antibody and by affinity chromatography with p13^{suc1}-Sepharose. Fig. 5*A* shows that all three anticytokinins significantly inhibited phosphorylation of histone H1 by immunopurified CDKs of *Arabidopsis* at concentrations between 10 and 100 μM. However, ANCYT3 was less effective and showed inhibitory activity only at 100 μM (Fig. 5*A*). Interestingly, G_2/M kinase CDKB1;1, immunoprecipitated with a specific antibody, proved to be much less sensitive to roscovitine and ANCYT1 (data not shown). Similar results were also obtained with human CDKs, where mitotic CDK1 is less sensitive to roscovitine and ANCYT3 than CDK2 (see below and Ref. 38). Inhibition by anticytokinins of *Arabidopsis* CDKs purified by p13^{suc1} binding was generally weaker than their inhibition of CDKs obtained by immunopurification; ANCYT1 was the most effective compound on CDKs purified by p13^{suc1} binding (Fig. 5*B*). The inhibitory effect of the compounds on p13^{suc1}-precipitated CDKs was observed only at the highest concentration tested (100 μM, Fig. 5*B*). p13^{suc1} is known to bind to several protein kinases (39), some of which might not be efficiently inhibited by the test compounds.

The amino acid composition of the ATP-binding pocket in CDKs is highly conserved among eukaryotic organisms (see supplemental Fig. S2). Therefore, to confirm the results obtained with *Arabidopsis* CDKs, we determined the inhibitory effect of anticytokinins on recombinant human CDK1-cyclin B (CDK1/B) and CDK2-cyclin E (CDK2/E). The data presented in Fig. 5, *C* and *D*, show that ANCYT1 inhibited both CDKs (10.5 μM in CDK1/B assays and IC_{50} = 5.2 μM in CDK2/E assays). ANCYT2 inhibited both CDKs as well, although somewhat less effectively (IC_{50} = 19.4 and 18.0 μM in CDK1/B and CDK2/E assays, respectively). ANCYT3 was only slightly active in the CDK1/B assay (IC_{50} = 44 μM), whereas CDK2/E was inhibited much more strongly (IC_{50} = 5.3 μM). These IC_{50} values are comparable with those previously described for the well established purine-based CDK inhibitors olomoucine (7 μM, CDK1/B; 5 μM, CDK2/E) but higher than those of roscovitine (0.45 μM CDK1/B; 0.2 μM, CDK2/E) (40, 41).

Next we studied the selectivity of kinase inhibition by these anticytokinins using a panel of diverse recombinant human kinases and appropriate phosphate-acceptor polypeptide substrates in the presence of 100 μM ATP. Supplemental Table 1 shows the measured IC_{50} values. The assay confirmed that ANCYT1 inhibits human CDK2 at low micromolar concentrations. The compound showed a similar potency toward both the mitogen-activated protein (MAP) kinase ERK2, which is phylogenetically closely related to CDKs, and GSK3, another kinase that is closely related to CDKs (42). CDK7 and CDK9 were also inhibited, albeit to a smaller extent. Similar differential effects have been shown for the CDK inhibitors olomoucine and roscovitine (42, 43).

The weight of the evidence reported above indicates that anticytokinins interact directly with CDKs, the most likely locus of this interaction being the highly conserved ATP-biding sites. To confirm this possibility, we determined the x-ray crystal structure of the complex between ANCYT1 and recombinant human CDK2. We chose human CDK2 because unlike plant CDKs, for which no structural information is currently available, its three-dimensional structure has been well characterized (41, 44–46). The human CDK2 is closely related to *Arabidopsis* CDKA;1. In fact, of the 27 residues that line the ATP-binding pocket of CDK2, all but one (Tyr-82 and Phe-82 in CDK2 and CDKA;1, respectively) are conserved (see supplemental Fig. S2). The structure of co-crystallized CDK2 and ANCYT1 shown in Fig. 6, *A–C*, reveals that the anticytokinin occupies the ATP-binding pocket of the enzyme (for structural data, see supplemental Table 2). Superimposition of ATP and ANCYT1 bound to CDK2 shows that the purine core structure of ANCYT1 occupies approximately the same space as the corresponding ring system in the substrate ATP but with different orientation (Fig. 6*D*). Thus the binding mode of ANCYT1 is similar to other CDK inhibitors, such as olomoucine, roscovitine, and purvalanol B (41, 45, 46).

The results of the inhibition and binding studies motivated us to compare the impact of anticytokinins and the CDK inhibitors olomoucine and roscovitine on the proliferation of human and plant cells. Fig. 7*A* shows the decrease of proportion of

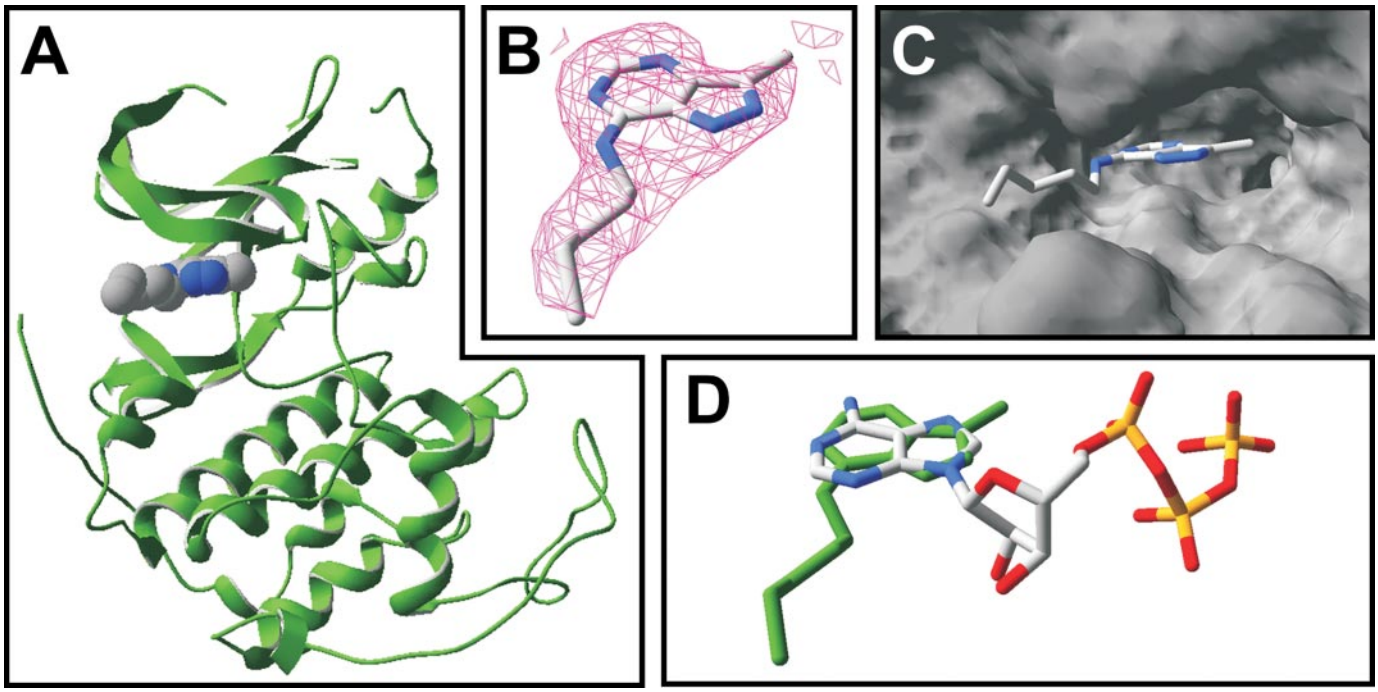

FIGURE 6. **X-ray crystal structure of the complex between recombinant human CDK2 and ANCYT1.** *A*, ANCYT1 (*space-filling Corey-Pauling-Koltun model*) binds between the N- and C-terminal lobes of CDK2 (*ribbon*). *B*, electron density calculated around ANCYT1 in the crystal structure ($2F_o - F_c$ contoured at 0.72 σ). *C*, binding mode of ANCYT1 (*CPK colors*) in the ATP pocket (*gray surface*). *D*, superimposition of the CDK2-bound ligands ATP (Protein Data Bank accession code 1HCK) (46) and ANCYT1.

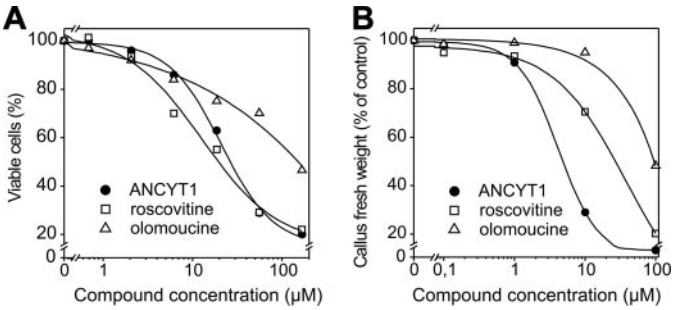

FIGURE 7. **Inhibition of cell proliferation.** *A*, dose-dependent inhibition of MCF7 cancer cell line proliferation by ANCYT1, roscovitine, and olomoucine. Viable cells were quantified by a calcein AM assay 72 h after compound application. Values are expressed as the percentage of cells grown without compounds. *B*, inhibitory effect of the anticytokinin ANCYT1, roscovitine, and olomoucine on tobacco callus growth induction by cytokinin (tZ). The tZ concentration was 0.5 μM, and the concentration of ANCYT1 ranged from 10 nM to 100 μM. Values are expressed as the percentage of average callus growth without ANCYT1 (12.9 g/flask). Average weights were calculated from five replicates, and the entire test was repeated twice.

viable cells of the breast cancer cell line MCF7 following exposure to ANCYT1, roscovitine, and olomoucine. ANCYT1 exhibited lower potency than the CDK inhibitor roscovitine but higher potency than olomoucine. ANCYT2 was less effective, and ANCYT3 had only a limited effect (data not shown). The inhibitory effect of ANCYT1 on the growth of two other cancer cell lines, K-562 and HOS, was comparable with the effect of olomoucine (data not shown).

The comparisons in plant cells were carried out with tobacco and *Arabidopsis* calli. Both cultures were grown on a medium containing 0.5 μM *trans*-zeatin, with concentrations of ANCYT1, roscovitine, or olomoucine ranging from 10^{-8} to 10^{-4} M. The growth of both callus cultures was inhibited following application of all three compounds. As shown in Fig. 7*B*, tobacco callus growth was inhibited more strongly by ANCYT1 (IC_{50} = 4.8 μM) than by roscovitine (IC_{50} = 26.7 μM) and olomoucine (IC_{50} = 95.4 μM). Weaker inhibitory activities were found in the *Arabidopsis* callus growth assay, but the IC_{50} values were of the same order (not shown).

DISCUSSION

We have analyzed the mode of action of several compounds that had previously been characterized as cytokinin antagonists and were proposed to act as competitive inhibitors at the cytokinin receptor (36). Interaction studies between three selected anticytokinins and two of the three recently identified *Arabidopsis* cytokinin receptors (CRE1/AHK4 and AHK3) revealed that these compounds neither activate the receptors in a bacterial assay nor inhibit the interaction of cytokinin with the receptors in receptor-enriched *E. coli* membranes (Fig. 2). Although these results do not eliminate the possibility that the anticytokinins might selectively inhibit AHK2, the third known *Arabidopsis* cytokinin receptor, which was not tested in our study, this possibility is considered to be unlikely; studied anticytokinins did not affect cytokinin activation of the primary response gene *ARR5* in *Arabidopsis* seedlings (Fig. 2*E*). Moreover, loss-of-function mutants of AHK2 show no growth defects, indicating functional redundancy of the receptors (6–8). Selective inhibition of AHK2 would therefore be expected to have little phenotypic consequence.

Taken together, our results favor an effect of anticytokinins on cytokinin action downstream of the initial receptor-cytokinin interaction. Indications of their mode of action came from similarities between anticytokinin-treated plant cells and cells treated with the CDK inhibitors olomoucine and roscovitine

(37). The compounds inhibited cell cycle progression at the G_2/M transition and caused abnormalities in microtubule structure. We subsequently demonstrated that ANCYT1, ANCYT2, and ANCYT3 inhibit the activity of purified *Arabidopsis* CDKA;1 and CDKB1;1 *in vitro*, although to different extents. Moreover, detailed studies showed that ANCYT1 binds to human CDK2 and occupies about the same space as the purine core structure of the kinase substrate ATP, albeit in a different orientation. Consistent with this, the representative anticytokinins inhibited cell proliferation more widely, not only in plant callus cultures of tobacco and *Arabidopsis*, the proliferation of which is cytokinin-dependent, but also in various human cancer cell lines, which proliferate independent of cytokinin (Fig. 7). Microscopic examination revealed that ANCYT1 disrupted mitotic processes. Abnormalities observed included the inhibition of nuclear envelope breakdown; the formation of dense prophase microtubules, which were randomly arranged around arrested nuclei; chromosome misalignment at the metaphase plate; absence of spindle bipolarity; and abnormalities during cytokinesis (Fig. 4). Abnormalities of the mitotic apparatus induced by ANCYT1 treatment could result from its inhibitory effect on CDKs and probably other related kinases that have functions during mitosis. The results are also in good agreement with the findings of Gregorini and Laloue (47), who previously demonstrated that in both cytokinin-requiring and cytokinin-autonomous tobacco cell suspension culture, only dividing cells are sensitive to the cytotoxic effects of ANCYT1.

It is of considerable interest that the anticytokinins had a similar activity in both plant and human cells. Cell cycle inhibition and induction of apoptosis are characteristic features of substances used for cancer treatment. The IC_{50} for CDK inhibition of the compounds investigated in this study are comparable with those of the cell cycle inhibitor olomoucine but higher than those of roscovitine. The latter compound is currently undergoing phase II clinical trials for the treatment of cancer based on its anti-proliferative and pro-apoptotic activities emanating from CDK inhibition (48). As small structural changes can have large effects on the activity of pyrazolo[4,3-*d*]-pyrimidines such as ANCYT1 (35), it may be possible to introduce structural modifications in ANCYT1 to improve its effectiveness in human cells. Moreover, although ANCYT1 is less effective than roscovitine on the activity of two human CDKs (Fig. 5, *C* and *D*), its ability to arrest growth of human cancer cells is comparable (Fig. 7*A*).

A further, similar cellular effect of ANCYT1 and roscovitine was observed, namely the induction of nuclear apoptotic changes. Data previously obtained by us showed that apoptotic changes were induced by roscovitine and olomoucine in a dose-dependent manner in plant cells.[5] Interestingly, although these drugs showed much stronger inhibitory effects on cell cycle progression than ANCYT1, the ability of roscovitine and olomoucine to induce apoptosis was weaker. Elucidation and comparison of the molecular mechanisms of apoptosis induction by anticytokinins and roscovitine will thus be of considerable interest.

[5] V. Cenklová, P. Binarová, L. Havliček, V. Kryštof, and M. Strnad, unpublished results.

Collectively, these findings strongly indicate that the inhibitory effect of anticytokinins is not directly related to cytokinin action at known receptors. Rather, their link with cytokinins appears to be a functional one concerned with their effects on the cell cycle, in which they appear to block cytokinin-dependent cell cycle progression. They were shown to inhibit CDKs and probably other cell cycle-associated kinases acting downstream of cytokinin receptors. In conclusion, we believe that it is appropriate to revise the classical view of anticytokinin mode of action, at least for the compounds tested here and related structures. The finding that anticytokinins also inhibit human CDKs and likely other kinases makes them interesting candidates for drug development.

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