Cooperative Antiproliferative Effects of 8-Chloro-Cyclic AMP and 528 Anti-Epidermal Growth Factor Receptor Monoclonal Antibody on Human Cancer Cells

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Abstract

8-Chloro-cyclic AMP (8-Cl-cAMP), a site-selective cAMP analogue, is a specific inhibitor of type 1 cAMP-dependent protein kinase (PKA) and induces growth inhibition in several human and rodent tumor cell lines. The anti-epidermal growth factor receptor (EGFR) mAb 528 is a blocking antibody able to inhibit the in vitro and in vivo growth of several human cancer cell lines that express functional EGFRs. Since enhanced levels of PKAI are generally found in tumor cells and an increase in PKAI expression is induced by transformation through a transforming growth factor α/EGFR autocrine pathway, we have evaluated whether treatment with mAb 528 in combination with 8-Cl-cAMP may have an additive or synergistic growth inhibitory effect on human cancer cells. A dose-dependent inhibition of monolayer cell growth was observed in two human colon cancer cell lines (GEO and CBS) and in a human breast cancer cell line (MDA-468) by treatment with either mAb 528 or 8-Cl-cAMP with 50% inhibitory concentration of 2–10 μg/ml or 20–25 nM, respectively. The combined treatment with low noninhibitory doses of mAb 528 (0.25 μg/ml) and with 8-Cl-cAMP has a more than additive growth inhibitory effect with a 3- to 5-fold reduction in the 8-Cl-cAMP 50% inhibitory concentration in all cell lines tested. This combined treatment was similarly effective in inhibiting the soft agar cloning efficiency of GEO cells. 8-Cl-cAMP treatment of GEO cells induced a dose-dependent increase in cell membrane-associated EGFRs with a maximum 3- to 4-fold increase within 48–72 h of treatment. These results suggest that a double blockade of the PKAI serine-threonine kinase-dependent and of the EGFR tyrosine kinase-dependent pathways is potentially useful in cancer therapy.

Introduction
cAMP³ acts in mammalian cells by binding to either of two distinct isoforms of PKA, defined as PKAI and PKAII (1). PKAI and PKAII share identical catalytic subunits, but differ in the regulatory subunits (termed RI in PKAI and RII in PKAII, respectively; Ref. 1). Differential expression of PKAI and PKAII has been correlated with cell differentiation and neoplastic transformation. In fact, preferential expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, while enhanced levels of PKAI are detected in tumor cells and in normal cells following exposure to mitogenic stimuli (2–5). In this respect, PKAI and/or its regulatory subunit RIα are generally overexpressed in human cancer cell lines and primary tumors and are induced following transformation by certain growth factors, such as TGF-α, or oncogenes, such as ras and erb-B2 (6–9). Furthermore, overexpression of RIα and/or PKAI has been recently shown as a marker of poor prognosis in breast cancer patients (10). For these reasons, PKA has been proposed as a potential target for cancer therapy (2). 8-Cl-cAMP, the most potent of a new class of site-selective cAMP analogues that discriminate between the two cAMP binding sites on RI and RII, is able to down-regulate RIα by facilitating the degradation of the protein while up-regulating at the transcriptional level RII expression (11–14). We have shown that down-regulation of RIα by 8-Cl-cAMP determines growth inhibition and differentiation in a variety of cancer cell lines in vitro and in vivo (11, 12). These effects are accompanied by an increased RII:RI ratio and by inhibition of different oncogenes and growth factors expression (6–9). Furthermore, we have recently completed a Phase I clinical trial of 8-Cl-cAMP administration in cancer patients refractory to standard therapy.⁴

Experimental and clinical studies have provided evidence for a TGF-α-mediated autocrine growth stimulation pathway in a number of human cancers (15). TGF-α acts by binding to the extracellular domain of the EGFR, thus activating its intracellular tyrosine kinase domain (15). Enhanced expression of TGF-α and/or EGFR has been detected in a majority of glioblastomas, breast, lung, ovarian, colorectal, and renal carcinomas (15). Furthermore, overexpression of EGFR has been as-

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associated with a poor prognosis in several human tumor types, such as breast cancer (16). Therefore, the blockade of the TGF-α/EGFR autocrine pathway through the use of specific anti-TGF-α neutralizing or anti-EGFR blocking mAbs has been proposed as a potential therapeutic modality (17, 18). In this respect, several blocking anti-EGFR mAbs that inhibit the in vitro and in vivo growth of human cancer cell lines have been generated (19–22). mAb 528 is a mouse IgG2a that binds to the EGFR with an affinity similar to that of EGF and TGF-α, competes with these ligands for receptor binding, and blocks EGF-induced or TGF-α-induced activation of EGFR tyrosine kinase (19). mAb 528 inhibits the in vitro proliferation of various human tumor cell lines which express TGF-α and EGFR (23). Furthermore, treatment with mAb 528 causes marked tumor growth inhibition in mice given s.c. injections of human cancer cell lines that express high EGFR levels (23, 24). In addition, it has been recently shown that the combined treatment of mice bearing well-established human tumor xenografts with mAb 528 or with mAb 225, a closely related anti-EGFR-blocking antibody, and with cytotoxic drugs, such as doxorubicin or cis-diamminedichloroplatinum, significantly increases the anti-tumor activity of these drugs (25, 26).

Our previous work has suggested a functional link between cell transformation involving a TGF-α/EGFR autocrine pathway and PKAI/RIa expression and activity (7, 8). This observation has prompted us to test whether the blockade of EGFR activation by mAb 528 treatment in combination with down-regulation of RIa by 8-Cl-cAMP treatment may be more effective than each treatment alone in inhibiting the growth of human cancer cell lines that express functional EGFRs. This approach has been recently reported (27). In this report we show that mAb 528 in combination with 8-Cl-cAMP determines a supraadditive growth inhibitory effect on human colon cancer (GEO and CBS) and breast cancer (MDA-468) cell lines.

Materials and Methods

Materials. The biochemical and biological characteristics of mAb 528, a mouse IgG2a mAb that binds to the EGFR with an affinity similar to that of EGF and TGF-α, competes with these ligands for receptor binding, and blocks the EGFR tyrosine kinase activation have been described previously (19, 20). 8-Cl-cAMP, a site-selective cAMP analogue (6), was kindly provided by Dr. K. Miki (Terumo Co., Saitama, Kana-gawa, Japan). Mouse 125I-EGF (specific activity, 100–120 μCi/μg) was purchased from Amersham Corp. (Milan, Italy).

Cell Cultures. GEO and CBS cell lines were kindly provided by Dr. M. Brattain (Baylor College of Medicine, Houston, TX). MDA-468 cells were a gift from Dr. C. Arteaga (Vanderbilt University, Nashville, TN). WIDR cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), 5 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml; Flow Laboratories, Irvine, Scotland), and insulin (10 μg/ml; Collaborative Biomedical Products, Bedford, MA) in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Monolayer Growth. To evaluate the effects of 8-Cl-cAMP and/or of mAb 528 on anchorage-dependent cell growth, cells (2 × 105 cells/well) were plated in 96-multiwell cluster dishes (Becton Dickinson, Milan, Italy) and treated every 48 h for three times with the indicated concentrations of 8-Cl-cAMP and/or of mAb 528. Cell growth was evaluated after 7 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method, as described previously (27).

Soft Agar Growth. Cells (5 × 103 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24-multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of 8-Cl-cAMP and/or of mAb 528. After 12 days the cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted as described previously (27).

Western Blot Analysis. Protein lysates (50 μg total protein/lane) were separated by SDS-PAGE (4–20% precast gradient gels; Bio-Rad Laboratories, Milano, Italy), transferred to nitrocellulose filters, and incubated with a 1:1000 dilution of an anti-human RIa mouse mAb, as previously described (9).

Flow Cytometric Analysis of Cell Cycle Distribution. Cells were trypsinized, washed twice with Ca2+/Mg2+-free PBS and fixed in 70% ethanol. Cells (106) were incubated at room temperature for 30 min in 1 ml of a propidium iodide staining solution (50 μl/ml in Ca2+/Mg2+-free PBS, pH 7.4). DNA analysis was performed in duplicate with a FACScan flow cytometer (Becton Dickinson) coupled with a Hewlett-Packard computer. Cell cycle data analysis was performed by the CELLFIT program (Becton Dickinson). Pulse area versus pulse width gating was performed to avoid doublets from the G2/M region.

Analysis of DNA Fragmentation. To evaluate the potential induction of programmed cell death by 8-Cl-cAMP and/or by mAb 528, cells were treated every 48 h with various concentrations of 8-Cl-cAMP and/or of mAb 528. After 96 h both adherent and detached cells were harvested, lysed, and DNA was extracted and electrophoresed as described (28).

125I-EGF Binding Assay. The binding assays were performed on cells in monolayer cultures using mouse 125I-EGF as described previously (29). The number of EGF binding sites and the Kd values for specific binding were determined by Scatchard analysis using the EBDA/LIGAND software for fitting multiple binding site data (29).

Results

As illustrated in Fig. 1, treatment with 8-Cl-cAMP induced a dose-dependent inhibition of monolayer growth in human breast (MDA-468) and colon (CBS and GEO) cancer cell lines, with an IC50 of 10, 25, and 25 μM, respectively. mAb 528 treatment was also able to induce a dose-dependent growth inhibition under the same culture conditions in all three cancer cell lines, with an IC50 of 10, 7, and 2 μg/ml, respectively. To determine whether the combined treatment with mAb 528 and 8-Cl-cAMP may have a cooperative effect on the anchorage-dependent growth, MDA-468, CBS, and GEO cells were treated with a low noninhibitory concentration of anti-EGFR mAb 528 (0.25 μg/ml) and with various concentrations of the cAMP analogue. This combination determined a more than additive antiproliferative effect with a 3- to 5-fold reduction in the 8-Cl-cAMP IC50 in all cell lines tested (Fig. 1). We next
Fig. 1 Effects of mAb 528 and/or 8-Cl-cAMP treatment on anchorage-dependent growth of MDA-468 (A), CBS (B), and GEO (C) human cancer cell lines. Two × 10^3 cells were plated in 96-multiwell cluster dishes and treated every 48 h, for three times with mAb 528 (0.25, 0.5, 1, 2, 5, or 10 μg/ml) or with 8-Cl-cAMP (0.25, 0.5, 1, 5, 10, or 25 μM) or with mAb 528 (0.25 μg/μl) plus 8-Cl-cAMP (0.25, 0.5, 1, 5, 10, or 25 μM). Cell growth was evaluated after 7 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Results represent the average (±SD) of three separate experiments, each performed in quadruplicate.

evaluated the effects of mAb 528 and/or 8-Cl-cAMP treatment on the anchorage-independent growth of GEO cells. As shown in Fig. 2A, both mAb 528 and 8-Cl-cAMP caused a dose-dependent reduction in soft agar cloning efficiency with an IC_{50} that was similar to that observed in monolayer cultures. Fur-

thermore, treatment with mAb 528 at 0.25 μg/ml in combination with 8-Cl-cAMP lowered the IC_{50} of the cAMP analogue from approximately 18 μM to 3 μM. To ascertain whether the supraadditive growth inhibitory effect of this combination could be obtained also with higher concentrations of mAb 528, GEO cells were treated in soft agar with 1, 2, or 5 μg/ml of the anti-EGFR blocking mAb in combination with 8-Cl-cAMP at 1 or 5 μM. Fig. 2B shows that a supraadditive inhibitory effect was obtained also in these conditions. For example, the combination of mAb 528 at 2 μg/ml and 8-Cl-cAMP at 1 μM determined an approximately 80% reduction in soft agar cloning efficiency,

Fig. 2 Effects of mAb 528 and/or 8-Cl-cAMP treatment on anchorage-independent growth of GEO cells. In A, 5 × 10^3/well were seeded in 24-multiwell cluster dishes in soft agar and treated with mAb 528 (0.25, 0.5, 1, 2, 5, or 10 μg/ml) or with 8-Cl-cAMP (0.25, 0.5, 1, 5, 10, or 25 μM) or with mAb 528 (0.25 μg/μl) plus 8-Cl-cAMP (0.25, 0.5, 1, 5, 10, or 25 μM). In B, cells were seeded in soft agar as above and treated with the indicated concentrations of 8-Cl-cAMP and/or mAb 528. After 12 days colonies larger than 0.05 mm were counted. The growth for untreated controls was 525 colonies/dish (A) and 545 colonies/dish (B). Results represent the average (±SD) of three separate experiments, each performed in triplicate.
Table 1  Cell cycle distribution of GEO cells following treatment with 8-Cl-cAMP and/or mAb 528

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell cycle distribution (%)</th>
<th>G0-G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>61</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>MAb 528 (µg/ml)</td>
<td></td>
<td>61</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td>61</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>60</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>54</td>
<td>37</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>55</td>
<td>37</td>
<td>8</td>
</tr>
<tr>
<td>8-Cl-cAMP (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>61</td>
<td>31</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>60</td>
<td>32</td>
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<tr>
<td>25</td>
<td></td>
<td>47</td>
<td>31</td>
<td>22</td>
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<tr>
<td>mAb 528 (0.25 µg/ml)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 8-Cl-cAMP (µM)</td>
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<tr>
<td>1</td>
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<td></td>
<td>42</td>
<td>38</td>
<td>20</td>
</tr>
</tbody>
</table>

*10° cells/35-mm dish were plated. Following 4 days of treatment with the indicated concentrations of 8-Cl-cAMP, the cells were trypsinized. Cell cycle distribution was assessed using propidium iodide DNA staining and analyzed with a FACScan flow cytometer. Data represent the average of three separate experiments. SD was less than 5%.

whereas each treatment alone determined a 45% or a 5% inhibition, respectively.

To evaluate whether 8-Cl-cAMP-induced and/or mAb 528-induced inhibition of GEO cell growth is correlated with a specific perturbation of the cell cycle, cell cycle analysis of GEO cells treated with different concentrations of the two compounds was performed. As compared to control untreated cells, an accumulation of GEO cells in G0-M was observed with the highest dose (25 µM) of 8-Cl-cAMP (Table 1). These results are in agreement with those of a previous study with human HL-60 leukemia cells (30). In contrast, no gross alteration in cell cycle distribution was detected when GEO cells were treated with the anti-EGFR mAb 528. In addition, the combined treatment of GEO cells with 0.25 µg/ml mAb 528 and with various concentrations of 8-Cl-cAMP did not change the effect of 8-Cl-cAMP on cell cycle distribution (Table 1). Similarly, the combination of higher doses of mAb 528, such as 1 or 5 µg/ml, and of 8-Cl-cAMP determined a perturbation of GEO cell cycle distribution which was comparable to that induced by 8-Cl-cAMP treatment alone (data not shown).

It has been suggested that growth factor deprivation may determine programmed cell death in several cell types (31). Furthermore, it has been recently shown that growth inhibition induced by anti-EGFR-blocking mAb treatment in human DCF colon cancer cells results in apoptosis (32). Therefore, we evaluated whether a similar phenomenon could occur in GEO cells following treatment with mAb 528 and/or 8-Cl-cAMP. The DNA from control GEO cells and from GEO cells treated for 96 h with mAb 528 (0.25, 1, 5, or 10 µg/ml), with 8-Cl-cAMP (1, 5, 10, or 25 µM), or with both was extracted and analyzed by agarose gel electrophoresis. No presence of chromatin fragmentation into nucleosome ladders was detected (data not shown). Therefore, in our experimental conditions the growth inhibition induced by the anti-EGFR-blocking antibody and/or by the cAMP analogue alone or in combination did not lead to evidence of apoptotic cell death.

We have previously shown that 8-Cl-cAMP treatment specifically induces a down-regulation of the RIα regulatory subunit of PKA1 in tumor cells (7–9). To determine whether mAb 528 could interfere with or potentiate this effect, GEO cells were treated for 4 days with different concentrations of 8-Cl-cAMP (1, 5, 10, or 25 µM), with mAb 528 (0.25 µg/ml), or with mAb 528 (1 µg/ml) plus 8-Cl-cAMP (1, 5, or 10 µM). A maximum of 50 to 80% reduction in RIα protein levels was observed using Western blotting in GEO cells treated with 10 or 25 µM 8-Cl-cAMP as compared to control untreated cells (Fig. 3, Lanes 1–5). In contrast, mAb 528 treatment alone did not affect RIα protein expression (Fig. 3, Lane 6). Furthermore, the combined addition of mAb 528 and 8-Cl-cAMP to GEO cells did not significantly increase the down-regulation of RIα protein levels as compared to GEO cells treated with equivalent concentrations of 8-Cl-cAMP alone (Fig. 3, Lanes 7–9).

Several studies have recently demonstrated that treatment of human cancer cells that possess functional EGFRs with cytokines, such as α-interferon, or with cytotoxic drugs, such as 1-b-d-arabinofuranosylcytosine and 5-aza-2'-deoxycytidine, at concentrations able to induce growth inhibition, is accompanied by an up-regulation of EGFR expression on the cell membrane (29, 33, 34). These findings have led to the hypothesis that treatment with pharmacological agents that inhibit tumor cell proliferation while up-regulating growth factor receptor expression may provide a new approach to improve the therapeutic index of antigrowth factor receptor antibodies or immunoconjugates (35). To determine whether 8-Cl-cAMP treatment could interfere with EGFR expression, we examined the specific 125I-EGF binding characteristics on GEO cells. As shown in Table 2, two classes of EGF binding sites with a high and a low affinity were detected by Scatchard analysis on untreated control GEO cells, which possess approximately 40,000 EGF receptors/cell. Treatment with 8-Cl-cAMP at a concentration of 5 or 10 µM for 72 h induced a 3- to 4-fold increase in EGF binding sites on GEO cells without any significant change in binding affinities or in the proportion of low and high affinity binding sites. To ascertain the kinetics of this EGFR up-regulation by the cAMP analogue, GEO cells were treated for various periods with different concentrations of 8-Cl-cAMP. A dose-dependent and a time-dependent up-regulation of EGFRs on GEO cells was observed with a maximum 3- to 4-fold increase following 48–72 h of treatment (Fig. 4).

Discussion

There is an extensive body of evidence demonstrating that human cancer cells express different families of growth factors, cytokines, and their cognate receptors, which contribute through intracellular, autocrine, paracrine, and juxtacrine pathways to regulate tumor growth, angiogenesis, and metastasis, and which can be involved in the development of tumor cell resistance to antineoplastic drugs (36). On these bases, efforts have been made to utilize the knowledge at a molecular level of growth factor receptor activation and of the intracellular growth factor-activated signal transduction pathways for the development of
Table 2 ¹²⁵I-EGF binding sites on GEO cells

<table>
<thead>
<tr>
<th></th>
<th>Sites/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High affinity</td>
</tr>
<tr>
<td>Control</td>
<td>6,600</td>
</tr>
<tr>
<td>8-Cl-cAMP (µM):</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7,900</td>
</tr>
<tr>
<td>5</td>
<td>25,000</td>
</tr>
<tr>
<td>10</td>
<td>23,500</td>
</tr>
</tbody>
</table>

* Treatment of GEO cells with 8-Cl-cAMP did not result in significant changes of K_I values. Values represent the average (±SD) of two independent experiments, each performed in triplicate. SD was less than 10%.

Fig. 3 Western blot analysis of Rlα protein expression. Lane 1, GEO cells, control; Lanes 2–5, GEO cells treated for 4 days with 8-Cl-cAMP (1, 5, 10, or 25 µM, respectively); Lane 6, GEO cells treated for 48 h with mAb 528 (0.25 µg/ml), Lanes 7–9, GEO cells treated with mAb 528 (0.25 µg/ml) plus 8-Cl-cAMP (1, 5, or 10 µM, respectively).

Fig. 4 Percentage of ¹²⁵I-EGF specifically bound to GEO cells treated with the indicated concentrations of 8-Cl-cAMP for the indicated periods as compared to control nontreated cells. Values represent the average (±SD) of two independent experiments, each performed in triplicate.
growth inhibition induced by the anti-EGFR-blocking mAb and/or by the site-selective cAMP analogue did not result in apoptotic cell death of GEO cells.

8-Cl-cAMP-induced cell growth inhibition is accompanied by a specific down-regulation of the RIIa regulatory subunit of PKA in tumor cells (7-9). Therefore, a potential mechanism by which 8-Cl-cAMP and mAb 528 may cooperate could be a more effective reduction in the RIIa protein levels. However, this is not the case since mAb 528 treatment alone did not affect RIIa protein levels in GEO cells. Furthermore, the combined addition of mAb 528 and 8-Cl-cAMP to GEO cells did not significantly increase the down-regulation in RIIa protein expression as compared to GEO cells treated with equivalent concentrations of 8-Cl-cAMP alone.

One of the mechanisms by which 8-Cl-cAMP exerts a supraadditive antiproliferative effect with the anti-EGFR blocking mAb 528 may be the up-regulation of EGFR expression on human cancer cells. In fact, a dose-dependent and a time-dependent up-regulation of EGFRs on GEO cells without any significant change in binding affinities or in the proportion of low and high affinity binding sites was observed with a maximum 3- to 4-fold increase following 48–72 h of treatment. A similar up-regulation in EGFR expression has been observed following treatment of human cancer cells that express functional EGFRs with other antineoplastic agents, such as doxorubicin, α-interferon, 1-β-d-arabinofuranosylcytosine, and 5-aza-2’-deoxycytidine (26, 29, 33, 34). This effect may have potential clinical relevance since it has been proposed that up-regulation of the EGFR on the tumor cell membrane could increase the tumor cell targeting and, therefore, the therapeutic index of anti-EGFR mAbs (35). In this respect, anti-EGFR-blocking mAbs or genetically engineered fusion proteins composed of an EGFR ligand and of a modified bacterial toxin, such as the TGF-α-PE40 Pseudomonas exotoxin fusion protein, have been shown to be generally more effective in inhibiting the cell growth of human cancer cell lines that express higher levels of functional EGFR (17, 18, 23, 37).

The results of this study provide the first experimental evidence for a supraadditive antiproliferative effect in human cancer cell lines in vitro of two agents that are able to specifically interfere with important steps of the tumor cell signal transduction machinery such as the EGFR tyrosine kinase and the PKA serine-threonine kinase. We are currently evaluating whether this effect could be obtained also in vivo against human tumor xenographs in immunodeficient mice and, therefore, whether the combination of these agents can be tested in cancer patients.

References


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