Activation of protein kinase A (PKA) by 8-Cl-cAMP as a novel approach for antileukaemic therapy

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Activation of PKA by cAMP agonists, such as 8-Cl-cAMP activation, selectively causes rapid apoptosis in v-abl transformed fibroblasts by inhibiting the Raf-1 kinase. Here we investigated whether 8-Cl-cAMP is useful for the treatment of chronic myelogenous leukaemia (CML), which is hallmarked by the expression of the p210bcr-abl oncogene. Autologous bone marrow transplantation is a feasible alternative for patients with no suitable donor, but hampered by the risk of relapse due to the persistence of leukaemia cells in the transplant. To study the effects of 8-Cl-cAMP on primary leukaemic cells, bone marrow cells (BMCs) from eight CML patients (one at diagnosis, three in chronic and four in accelerated phase) were treated. Ex vivo treatment of BMCs obtained in chronic phase of CML with 100 μM 8-Cl-cAMP for 24–48 h led to the selective purging of Philadelphia Chromosome (Ph1 chromosome) without toxic side effects on BMCs from healthy donors as measured by colony-forming unit (CFU) assays. BMCs from patients in accelerated phase showed selective, but incomplete elimination of Ph1 chromosome positive colony forming cells. The mechanism of 8-Cl-cAMP was investigated in FDCP-mix cells transformed by p210bcr-abl, a cell culture model for CML. The results showed that 8-Cl-cAMP reduced DNA synthesis and viability independent of Raf inhibition as Raf inhibitors had no effect. MEK inhibitors interfered with DNA synthesis, but not with viability. In summary, our results indicate that 8-Cl-cAMP could be useful to purge malignant cells from the bone marrow of patients with CML and certain other forms of leukaemias.

Keywords: leukaemia; purging; protein kinase A; 8-Cl-cAMP

The activation of signalling pathways due to the constitutive expression of the Bcr-Abl oncogene plays a major role in the pathogenesis of some leukaemias, in particular chronic myelogenous leukaemia (CML). CML is the major adulthood leukaemia characterised by the Philadelphia chromosome (Ph1 chromosome, t:9/22), a chromosomal translocation where bcr sequences from chromosome 22 are juxtaposed to c-abl on chromosome 9 leading to the expression of an atypical fusion-protein p210bcr-abl (Butturini et al, 1996). In its chronic phase CML is hallmarked by abnormally sustained cell survival rather than excessive proliferation and is relatively well controlled by cytoreductive chemotherapy. However, the chronic phase inevitably turns into an acute phase of blast crisis where leukaemic blast cells proliferate rapidly and aggressively with fatal consequences.

p210bcr-abl is a constitutively activated tyrosine kinase that activates numerous cellular signalling pathways including the Raf–MEK–ERK pathway, which is critical for malignant transformation. A tyrosine kinase inhibitor for p210bcr-abl, CGP-57148 now called STI571, has sparked great interest as it dramatically increased the number of patients achieving complete remission. However, almost half of the patients treated in the chronic phase remain Ph1 chromosome positive with the inherent risk of relapse. Indeed, many patients treated with STI571 in the acute phase relapse rapidly (La Rosee et al, 2002). This probably relates to the fact that STI571 is inhibiting proliferation rather than eliminating the leukaemic cells (Beran et al, 1998; La Rosee et al, 2002). Despite the addition of STI571 to the clinical arsenal bone marrow and peripheral blood stem cell transplantation (PBSCT) remains a mainstay of therapy. For autologous PBSCT stem cells are harvested from peripheral blood after stimulation with G-CSF at the time of clinical remission. A proportion of patients responds at least initially to autologous PBSCT (Reiffers et al, 1994). Regardless whether PBSCT or autologous bone marrow transplantation is used, it is crucial to eliminate leukaemic cells from the transplant in order to avoid the transfer of leukaemic cells back to the patient.

For CML the logical target that distinguishes normal cells from leukaemic cells is p210bcr-abl. This fusion protein aberrantly activates a number of signalling pathways that act in concert to transform cells. Thus, these pathways emanating from p210bcr-abl are all potential targets for therapeutic intervention. Prominent
targets are the Raf-1 kinase and the c-Myc transcription factor. p210<sup>c</sup>-<i>abl</i> induces c-Myc expression in haematopoietic cells (Sawyers et al., 1992; Sawyers, 1993; Weissinger et al., 1993). The deregulation of c-Myc expression has been shown to be required for transformation by oncogenic abl genes (Sawyers, 1993; Weissinger et al., 1993). p210<sup>c</sup>-<i>abl</i> also activates Raf-1 and the MEK–ERK pathway. The canonical Raf-MEK–ERK pathway is often perceived as a linear signalling module that mediates cell proliferation, transformation, and survival (Weissinger et al., 1997).

We have shown previously that the activation of the cAMP dependent protein kinase A (PKA) with synthetic agonist drugs such as 8-Chloro-cyclic Adenosine Monophosphate (8-Cl-cAMP) results in the inhibition of Raf-1 kinase activity and rapid apoptosis induction in v-abl transformed fibroblasts. Apoptosis occurred despite a high constitutive activity of ERK suggesting that Raf-1 uses a different pathway to ensure cell viability (Weissinger et al., 1997).

These observations led us to explore the use of 8-Cl-cAMP for the treatment of CML bone marrow cells for ex vivo for the purging of leukaemic bone marrow or for the treatment of leukaemic patients. 8-Cl-cAMP is one of the most stable compounds that activate PKA (Schwed et al., 2000) and can be manufactured in quantities and quality sufficient for clinical use. In fact, the antitumour activity of 8-Cl-cAMP has been under study for a number of years, including clinical studies for the treatment of tumours (Cho-Chung et al., 1999; Tortora et al., 1995; Proper et al., 1999). To date, mainly solid tumours like breast carcinomas were studied. In this report we present data indicating that treatment with 8-Cl-cAMP can provide an effective method for purging bone marrow prior to autologous transplantation, targeting specifically the p210<sup>c</sup>-<i>abl</i> transformed cells.

**PATIENTS AND METHODS**

**Patients**

The studies were approved by the institutional ethics committees of Munich and Hannover. Bone marrow from healthy donors, eight patients with CML (five males and three females age range: 25–49) in haematological chronic phase or at more advanced stages was obtained after informed consent.

**Synthesis of 8-Cl-cAMP**

Preparation of 8-Cl-cAMP was performed as described with minor modifications (Brentnall and Hutchinson, 1972; Schwed et al., 2000). Briefly, 30 g (85.47 mmol) cAMP, sodium salt, were suspended in 2000 ml DMF and reacted with 87 g (171 mmol) 8-Cl-cAMP was added to the cultures as indicated on day 0 and incubated in 0.075 mol l<sup>-1</sup> -minimal medium or 1% HS. The cells were resuspended in LTC-medium supplemented with 100 μl of a colchicine solution (1 μg ml<sup>-1</sup> in χ-minimal essential medium) to 1 ml of cell suspension for 12 h. The cells were subsequently transferred to poly-L-lysine-coated slides and incubated in 0.2 ml of 0.075 mol l<sup>-1</sup> KCl at room temperature for 10 min. Cells were fixed by gently dropping 100 μl cold methanol on the slide. The excess of the fixative was removed with absorbent paper and slides were dried on a hot plate at 55 °C. After repeated cold fixation for 15 min, the cells were used for banding and cytogenetic analyses.

**Cytogenetic analyses**

After completion of the CFU assays, colonies were picked and analysed for Ph1 chromosome positive colonies. Cytogenetic analyses were essentially performed as described (Dube et al., 1981). Briefly, cell division was arrested in metaphase by the addition of 100 μl of a colchicine solution (1 μg ml<sup>-1</sup> in χ-minimal essential medium) to 1 ml of cell suspension for 12 h. The cells were subsequently transferred to poly-L-lysine-coated slides and incubated in 0.2 ml of 0.075 mol l<sup>-1</sup> KCl at room temperature for 10 min. Cells were fixed by gently dropping 100 μl cold methanol on the slide. The excess of the fixative was removed with absorbent paper and slides were dried on a hot plate at 55 °C. After repeated cold fixation for 15 min, the cells were used for banding and cytogenetic analyses.

**Growth curves**

8-Cl-cAMP was added to the cultures as indicated on day 0 and was not replenished during the culture. FDCP-mix cells were plated in 24-well plates at a density of 2 × 10<sup>5</sup> cells ml<sup>-1</sup> in LTC medium as described above in the presence or absence of 8-Cl-cAMP for 24 h. Subsequently, the cells were washed and grown in long-term culture medium. In total, 2 × 10<sup>5</sup> cells ml<sup>-1</sup> were used for CFU assays. At the end of the long-term culture (4–6 weeks), the adherent cells (ADC) as well as the NADC were harvested and analysed in the same manner.

**Nonspecific toxicity testing of 8-Cl-cAMP**

The nonspecific toxicity of 8-Cl-cAMP on bone marrow cells was tested using marrow mononuclear cells (MNC) from healthy donors as outlined in Figure 1. The MNCs were cultured at a density of 2 × 10<sup>5</sup> cells ml<sup>-1</sup> in LTC medium as described above in the presence or absence of 8-Cl-cAMP for 24 h. The cells were washed and grown in long-term culture medium. In total, 2 × 10<sup>5</sup> cells ml<sup>-1</sup> were used for CFU assays. At the end of the long-term culture (4–6 weeks), the adherent cells (ADC) as well as the NADC were harvested and analysed in the same manner.

**Viability assay**

FDCP-mix cells were washed and resuspended (4 × 10<sup>5</sup> cells ml<sup>-1</sup>) in Fisher’s medium supplemented with 20% (v/v) FCS and 10<sup>-1</sup> hydrogen peroxide at a density of 2 × 10<sup>6</sup> cells ml<sup>-1</sup> and cultured for 0 to 24 h. Samples were taken after 24, 48 and 72 h in culture. Cell viability was analysed by flow cytometry using the annexin V-FITC, propidium iodide (PI) based assay (R&D Systems, Oxford, UK) as previously described (Francis et al., 2000). Samples were analysed using a FACSVantage flow cytometer (Becton Dickinson Co., Mountain View, CA, USA). Viable cells (unstained), early apoptotic cells and necrotic cells were analysed. Results are shown ± s.e.m. (n = 3).
RESULTS

PKA-activation resulted in a transient growth inhibition of human bone marrow cells

The nonspecific toxicity of 8-Cl-cAMP was tested on human bone marrow cells (BMC) of healthy donors (Table 1). In total, 15 flasks of cells per donor were set up for long-term cultures after treatment with 8-Cl-cAMP, five for each condition (control, 50 µM and 100 µM 8-Cl-cAMP; Figure 1). BMCs taken at week 0 immediately after treatment with 8-Cl-cAMP showed a reduction of cell numbers and a concomitant reduction of colonies arising in the CFU assays. However, at all subsequent timepoints, ranging from 1 to 5 weeks, comparable numbers of CFU-initiating cells were obtained from treated and untreated BMC cultures. This is summarised in Table 1 showing CFUs obtained from week 5 cultures as example. Thus, 8-Cl-cAMP only caused a transient impairment of CFU capacity in BMCs from healthy donors that was readily reversed at longer time points. This is consistent with our earlier observations (Weissinger et al., 1997) that untransformed cells only respond to PKA activation with an initial, transient inhibition of proliferation.

Ph1 chromosome positive colony forming cells are selectively eliminated by 8-Cl-cAMP

Since no severe nonspecific toxicity was observed that would prohibit treatment with 8-Cl-cAMP, bone marrow MNCs of eight patients with CML were treated in the same manner (Table 2). Three patients were in the chronic phase of CML undergoing cytoreductive treatment, one patient was at diagnosis and four were in the accelerated phase or blast crisis. In all cases, treatment with 8-Cl-cAMP substantially reduced the number of Ph1 chromosome positive colonies. The incubation with 50 µM and 100 µM 8-Cl-cAMP for 24 h immediately after treatment with 8-Cl-cAMP showed a reduction of cell numbers and a concomitant reduction of colonies arising in the CFU assays. However, at all subsequent timepoints, ranging from 1 to 5 weeks, comparable numbers of CFU-initiating cells were obtained from treated and untreated BMC cultures. This is summarised in Table 1 showing CFUs obtained from week 5 cultures as example. Thus, 8-Cl-cAMP only caused a transient impairment of CFU capacity in BMCs from healthy donors that was readily reversed at longer time points. This is consistent with our earlier observations (Weissinger et al., 1997) that untransformed cells only respond to PKA activation with an initial, transient inhibition of proliferation.

Table 1 Colony formation assays and cytogenetic analyses on normal volunteer marrow MNC

| Cultures          | Granulocytes (G) | Eosinophiles (Eo) | Macrophages (M) | Erythrocytes (E) | Mixed colonies (GM) | Blasts | Total no. |
|-------------------|------------------|-------------------|-----------------|-----------------|---------------------|--------|-----------|
| Week 0 control   | 49               | 2                 | 61              | 128             | 12                  | 7      | 259       |
| 8-Cl-cAMP (100 µM)| 27               | 4                 | 18              | 100             | 7                   | 5      | 145       |
| Week 5 control   | ADC              | 2                 | 16              | 6               | 2                   | 5      | 88        |
| 8-Cl-cAMP (100 µM)| ADC              | 2                 | 10              | 30              | 14                  | 22     | 148       |

Data obtained after treatment of normal marrow MNC with 8-Cl-cAMP are summarised. The cells were resuspended at 2.5 x 10⁶ cells ml⁻¹ and incubated at 50 µM 8-Cl-cAMP for 24 h as indicated. After 24 h cells were washed, and expanded in long-term cultures as described. CFU assays were setup weekly with the nonadherent cells (NADC). At the end of the long-term culture, adherent cells (ADC) and nonadherent cells (NADC) were harvested and analysed in separate CFU-assays.

Table 2 Percentage Philadelphia chromosome positive colonies (CFU-GM) in patients with CML in the presence or absence of 8Cl-cAMP

| Patient ID | Control | 8Cl-cAMP 50 µM | 8Cl-cAMP 100 µM | Control | 8Cl-cAMP 50 µM | 8Cl-cAMP 100 µM |
|------------|---------|----------------|----------------|---------|----------------|----------------|
| 1216 (cp)  | 100 (2/2)| 100 (20/20)    | 100 (28/28)    | ND      | 14 (3/21)      | 0 (0/15)       |
| 1206 (cp)  | 100 (12/12)| ND             | 70 (12/17)     | 83 (30/36)| ND             | 0 (0/15)       |
| 1726 (cp)  | 100 (62/62)| ND             | 100 (25/25)    | 83 (26/29)| ND             | 0 (0/37)       |
| 166 (D)    | 100 (6/6) | 100 (15/15)    | ND             | 100 (20/20)| 46 (7/15)       | ND             |
| A97/3 (ap) | 100 (21/21)| ND             | 100 (30/30)    | 100 (5/5) | ND             | 83 (5/6)       |
| 371 (ap)   | 81 (27/33)| ND             | 92 (24/26)     | 81 (27/33)| ND             | 33 (1/3)       |
| 361 (ap)   | 100 (30/30)| ND             | 100 (19/19)    | 100 (30/30)| ND             | 50 (2/4)       |
| 341 (ap)   | 100 (40/40)| ND             | 96 (26/27)     | 100 (34/34)| ND             | 62 (5/8)       |

The Ph1 chromosome positive colonies (in %) arising from patient bone marrow cells in percent are summarised, the actual number of colonies is given in parenthesis (Ph1 chromosome+total number). MNC were incubated for 24 h without or with 50 or 100 µM of 8-Cl-cAMP as indicated, washed and resuspended in long-term culture medium (Dexter). CFU- assays were setup in week 0 and weekly thereafter. CFU-GM of the adherent fraction of the cells (ADC) is shown, since there were only few colonies in the nonadherent fraction at this time. Ph1 chromosome status was determined by cytogenetic analysis. cp = chronic phase; D = diagnosis; ap = accelerated phase; ND = not determined.
8-Cl-cAMP resulted in a reduction of Ph1 chromosome positive colonies to 14 and 46%, respectively, but never led to a complete elimination of the Ph1 chromosome positive progenitor cells. Therefore, 100 μM 8-Cl-cAMP was used in further experiments.

In CFU assays prepared from cultures at week 0 all colonies were Ph1 chromosome positive, and no difference was observed between the treated and the untreated cells. However, treatment with 8-Cl-cAMP resulted in a significant reduction of Ph1 chromosome positive CFUs prepared after 5 weeks of culture depending on the stage of disease. In three patients undergoing cytoreductive therapy, the treatment with 100 μM 8-Cl-cAMP led to a complete loss of Ph1 chromosome positive colonies after 5 weeks of culture. In the absence of 8-Cl-cAMP only a small reduction of Ph1 chromosome positive colonies was observed after 5 weeks of culture, with more than 80% of the colonies remaining Ph1 chromosome positive. Cells from one untreated patient and four patients in advanced stages of CML exhibited a reduction of cells with a significant loss of Ph1 chromosome positive colonies ranging from 83 to 33%. Interestingly, after treatment with 8-Cl-cAMP Ph1 chromosome negative colonies appeared in CFU assays prepared from patients in the accelerated phase of CML, whereas in the untreated controls 100% of the colonies were Ph1 chromosome positive.

Studies to investigate the molecular mechanism of 8-Cl-cAMP

Given the encouraging results obtained with 8-Cl-cAMP in clinical samples and patients we investigated the molecular basis of the activity of 8-Cl-cAMP in particular with regard to its effects on cell proliferation and survival. Due to technical reasons such as freshness and instability of the material, these studies are extremely difficult in primary clinical samples. Therefore, we used bcr-abl transformed FDCP-mix p210_bcr-abl cells, a well characterised cell culture model system for CML (Pierce et al., 1998). These cells are conditionally transformed by expression of a temperature-sensitive p210_bcr-abl protein. They still remain IL-3 dependent although p210_bcr-abl sensitisises them to the effects of IL-3, when cultured at the permissive temperature of 32°C. The main effect of p210_bcr-abl is to enhance viability under conditions of low IL-3 levels (0.01 – 0.1 ng ml⁻¹) (Pierce et al., 1998).

Our previous studies with v-abl transformed fibroblasts (Weissinger et al., 1997) had indicated that PKA activation could downregulate the activity of Raf-1 and thereby cause apoptosis in these cells. This would provide a plausible explanation for the effects of 8-Cl-cAMP on CML cells. To test whether this hypothesis was also applicable to haematopoietic cells we compared 8-Cl-cAMP to selective pharmacological inhibitors of Raf-1 and MEK. As observed previously (Pierce et al., 1998) p210_bcr-abl did not significantly affect DNA synthesis when cells were compared to parental controls within 24 h after shifting them to the permissive temperature 32°C (Figure 2). The two Raf kinase inhibitors (RafKI and ZM336372) failed to interfere with IL-3 driven proliferation. RafKI blocked a accelerated proliferation in both the control cells and p210_bcr-abl cells exposed to 10 ng ml⁻¹ IL-3. In contrast, both MEK inhibitors (U0126 and PD98059) interfered with DNA synthesis and this effect was slightly more pronounced in the p210_bcr-abl cells. In control cells 8-Cl-cAMP interfered with DNA synthesis only at high (10 ng ml⁻¹) concentrations of IL-3, whereas it blocked proliferation in p210_bcr-abl cells at all concentrations.

A clear difference emerged when the effects on viability were assayed using trypan blue exclusion (Figure 3). FDCP-mix cells have been reported to die by apoptosis following cytokine removal (Williams et al., 1990). p210_bcr-abl protected cells from the cytotoxic effects of IL-3 withdrawal, maintaining the viability of almost 40% of cells 3 days after IL-3 withdrawal. Under these conditions the viability of the control cells was severely compromised. IL-3 was a very potent survival factor even at 0.1 ng ml⁻¹. Higher concentrations of IL-3 did not improve survival further. Interestingly, neither MEK inhibitors (Figure 3A) nor Raf inhibitors (Figure 3B) counteracted effects of p210_bcr-abl or IL-3 on cell viability.

In contrast, 8-Cl-cAMP significantly inhibited the cytotoxic effect of p210_bcr-abl, but not of IL-3 (Figure 4A). Moreover, 8-Cl-cAMP preferentially induced cell death in p210_bcr-abl as compared to control cells. This effect was most pronounced 48 and 72 h after IL-3 withdrawal, suggesting that p210_bcr-abl sensitisises cells to killing by 8-Cl-cAMP. IL-3 protected against 8-Cl-cAMP induced cytotoxicity suggesting that IL-3 can activate p210_bcr-abl independent survival pathways. As trypan blue exclusion (Figure 4A) does not distinguish between necrotic and apoptotic cell death, we further tried to dissect the mode of 8-Cl-cAMP induced cell death. Apoptosis leads to cell surface phospholipid asymmetry resulting in the exposure of phosphatidylserine (PS) on the outer leaflet of the cytoplasmic membrane. Annexin V preferentially binds PS and has been used to detect apoptosis in the FDCP-mix cells (Francis et al., 2000). In contrast to necrosis, membrane integrity is maintained during apoptosis precluding staining of DNA by the membrane impermeable dye propidium iodide (PI). Thus, apoptosis is indicated by positive staining for annexin V and negative staining for PI. As shown in Figure 4B measuring viability as cells that escape apoptosis, that is, stain negative for annexin, largely parallels the data obtained with the trypan blue exclusion assay, exception that in this assay the parental FDCP-mix cells also show a significant decrease in viability in response to 8-Cl-cAMP 48 and 72 h after IL-3 removal. Measuring annexin positive and PI negative, that is, apoptotic cells (Figure 4C) showed a higher rate of
Significant increases were observed in control cells 24 h, and in the enhanced by 8-Cl-cAMP under conditions of IL-3 withdrawal. The initial decline in cell numbers is probably due to the inhibition of proliferation also observed in untransformed NIH3T3 fibroblasts or even in v-raf transformed fibroblasts (Weissinger et al, 1997). The reversible inhibition of normal cell proliferation could potentially be exploited to protect stem cells during chemotherapy. Thus, combining 8-Cl-cAMP with classical DNA damaging chemotherapeutic drugs may have the added benefits of assaulting the leukaemic cells by two routes while protecting the stem cells at the same time.

Seeking to understand the mechanism of growth inhibition and apoptosis induced in patient cells, we employed a well-characterised cell culture model of CML, that is, FDCP-mix cells expressing a temperature-sensitive p210<sup>bcr/abl</sup> oncogene. We have previously shown that the inhibition of Raf-1 by 8-Cl-cAMP led to apoptosis in v-abl transformed fibroblasts, while control cells or cells expressing the v-raf oncogene showed only a reversible growth inhibition (Weissinger et al, 1997). Here we demonstrate that the activation of PKA is a promising approach to selectively eliminate the Ph1 chromosome positive progenitor cells from marrow obtained from CML patients. Despite an initial reduction of total cell numbers no long-term cytotoxicity was observed when marrow cells from normal donors were treated with 50 or 100 µM 8-Cl-cAMP (Table 1). The initial decline in cell numbers is probably due to the inhibition of proliferation also observed in untransformed NIH3T3 fibroblasts or even in v-raf transformed fibroblasts (Weissinger et al, 1997). The reversible inhibition of normal cell proliferation could potentially be exploited to protect stem cells during chemotherapy. Thus, combining 8-Cl-cAMP with classical DNA damaging chemotherapeutic drugs may have the added benefits of assaulting the leukaemic cells by two routes while protecting the stem cells at the same time.

In summary, the inhibitor experiments demonstrated that the pathways mediating proliferation can be dissociated from pathways mediating apoptosis. MEK – ERK signalling is required for DNA synthesis, but not for viability, whereas 8-Cl-cAMP can interfere with cell proliferation as well as survival. More importantly they show that 8-Cl-cAMP preferentially kills p210<sup>bcr/abl</sup> cells.

**DISCUSSION**

In this report, we have analysed the influence of PKA-activation on transformed cells from eight patients with CML. The expression of p210<sup>bcr/abl</sup> is a hallmark of CML. Among other signalling pathways p210<sup>bcr/abl</sup> also activates the Raf – MEK – ERK pathway. We have previously shown that the inhibition of Raf-1 by 8-Cl-cAMP led to apoptosis in v-abl transformed fibroblasts, while control cells or cells expressing the v-raf oncogene showed only a reversible growth inhibition (Weissinger et al, 1997). Here we demonstrate that the activation of PKA is a promising approach to selectively eliminate the Ph1 chromosome positive progenitor cells from marrow obtained from CML patients. Despite an initial reduction of total cell numbers no long-term cytotoxicity was observed when marrow cells from normal donors were treated with 50 or 100 µM 8-Cl-cAMP (Table 1). The initial decline in cell numbers is probably due to the inhibition of proliferation also observed in untransformed NIH3T3 fibroblasts or even in v-raf transformed fibroblasts (Weissinger et al, 1997). The reversible inhibition of normal cell proliferation could potentially be exploited to protect stem cells during chemotherapy. Thus, combining 8-Cl-cAMP with classical DNA damaging chemotherapeutic drugs may have the added benefits of assaulting the leukaemic cells by two routes while protecting the stem cells at the same time.

Seeking to understand the mechanism of growth inhibition and apoptosis induced in patient cells, we employed a well-characterised cell culture model of CML, that is, FDCP-mix cells expressing a temperature-sensitive p210<sup>bcr/abl</sup> oncogene. We have previously shown that the inhibition of Raf-1, but not MEK, would be crucial for the cytotoxic effects of 8-Cl-cAMP. Therefore, we compared the effects of 8-Cl-cAMP to Raf-1 and MEK inhibitors. The results clearly show that MEK activity is required for the proliferation of both normal and p210<sup>bcr/abl</sup> cells. In contrast, MEK activity was not required for p210<sup>bcr/abl</sup> or IL-3 mediated viability. Curiously, Raf-1 inhibitors did not inhibit proliferation or survival, and Raf KI even enhanced these parameters. These results suggest that Raf-1 does not play a significant role in mediating proliferation or survival in these cells. However, the unexpected effects of Raf kinase inhibitors may be explained by a paradoxical activation of Raf previously observed with ZM 336372 (Hall-Jackson et al, 1999).

Alternatively, Raf kinase activity may be dispensable for maintenance of cell viability, as Raf-1 has been shown to prevent apoptosis independent of its kinase activity by binding to and inhibiting the activity of the proapoptotic kinase ASK-1 (Chen et al, 2001). In this scenario, the Raf inhibitors would not be expected to show any effects, since they are ATP analogues that block kinase activity but not binding to other proteins. Furthermore, myeloid cells can activate the ERK pathway independent of Raf (Buscher et al, 1995), which could explain why the Raf and MEK inhibitors have different effects. Thus, the inhibitory effects of 8-Cl-cAMP on the proliferation and viability of p210<sup>bcr/abl</sup> cells cannot be explained by the inhibition of the catalytic activities of Raf-1 and MEK.

Importantly, 8-Cl-cAMP exhibited significant selective cytotoxicity for cells that express p210<sup>bcr/abl</sup>. This was shown with the p210<sup>bcr/abl</sup> transformed FDCP-mix cells as well as with primary bone marrow cells from leukaemic and normal donors. When marrow was obtained from patients in chronic phase of CML, a single incubation with 100 µM 8-Cl-cAMP for 24 h was sufficient to
Figure 4  Analysis of the effect of 8-Cl-cAMP on the viability of FDCP-mix and p210^{bcr-abl} transformed FDCP-mix cells. Cells were cultured as in Figure 2. IL-3 was removed and 8-Cl-cAMP (100 µM) was added. Cell viability was assessed 24, 48 and 72 h after IL-3 removal using (A) trypan blue exclusion, (B–D) Annexin and propidium iodide staining as described in the Materials and Methods section. Experiments were carried out in triplicates. The significance of changes was analysed by Student’s paired T-test and significant changes are indicated in the figure along with the P-values.

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