Anti-Cancer Drugs 1997, **8**, pp. 1007–1013

Case report

Induction of apoptosis and regression of spontaneous dog melanoma following *in vivo* application of synthetic cyclin-dependent kinase inhibitor olomoucine

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This case report describes a dog with spontaneous melanoma of the orofacial region which was treated by a synthetic inhibitor of cyclin-dependent kinases, i.e. olomoucine (OC). The drug was applied i.v. in a single dose of 8 mg/kg/day for 7 days in succession. Repeated bioptic examinations of metastatic cervical lymph nodes showed rapid induction of apoptosis in tumor cells as early as on the third day of treatment. Standard clinical and laboratory examinations did not reveal side effects of the therapy. There were no detectable manifestations of myelosuppression, hepatotoxicity, nephrotoxicity or neurotoxicity. However, transient anemia developed following bleeding from a devitalized tumor mass. For this reason, the dog underwent surgery to minimize tumor load as well as to eliminate the source of bleeding. Two kilograms of primary tumor were extirpated in the course of surgery, including cervical node metastases. Unfortunately, the dog died soon after surgery due to respiratory depression. Histological examinations of the tumor tissue showed marked apoptosis of melanoma cells in both the primary tumor and metastases. The induction of programmed cell death of cancer cells by OC resulted in rapid eradication of at least 68% of the tumor cells. The remaining melanoma cells retained at least equally well *in vitro* sensitivity to OC as to drugs currently used in clinical practice.

This project was supported, in parts, from the Grant Agency of Czech Republic (no. 204/96/K231 and 301/96/K047), the Grant Agency of Palacký University (no. 14501103), the League Against Cancer Prague and from the profit of Terry Fox Run organized by the Canadian Embassy in Prague.

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Key words: Apoptosis, chemotherapy, cyclin-dependent kinase inhibitor, malignant melanoma, olomoucine.

Introduction

A few years ago, it was shown that a family of cell cycle regulatory proteins designated 'mitotic inhibitors' or cyclin-dependent kinase (CDK) inhibitors (CDKI) plays a major role in regulation of progression throughout the cell cycle.[1] These include p21, the levels of which are elevated upon DNA damage in G_1 in a p53-dependent manner,[2] a related protein designated p27, which functions as a negative regulator of G_1 progression and may function as a possible mediator of transforming growth factor (TGF)-β-induced G_1 arrest,[3] and a more recently identified protein designated p57.[4] An additional mitotic inhibitor, p16, functions in part by binding cdk4/cdk6 and thereby inhibits the catalytic activity of cdk4/cdk6 cyclin D complexes on the G_1/S boundary.[5] A more recently isolated member of the p16 family has been designated p15. Protein p15 expression is up-regulated approximately 30-fold in TGF-β-treated human keratinocytes, suggesting that p15 may act as an effector of TGF-β-mediated cell cycle arrest.[6] The recent identification of additional mitotic inhibitory proteins, p18[7] and p19,[8] suggests that this may be a larger family of proteins than initially realized.

As mentioned above, mitotic inhibitory proteins exert their biological activity via inhibition of selected

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CDKs. The fact that these proteins themselves or the genes controlling their transcription, e.g. p53, are frequently mutated in cancer cells,[9] suggests that mitotic inhibitors may play a role in tumorigenesis and that manipulation of their activity may provide a logical means of treating neoplasia. Recently it has been shown that a retroviral vector containing a gene encoding one CDKI, p21, can inhibit proliferation and induces differentiation of tumor cells *in vivo*.[10]

Another way to brake the cell cycle engine in cancer cells is to mimic the functions of altered CDKIs in the down-regulation of CDK activity. In order to investigate this approach, we used of synthetic inhibitor of CDKs called olomoucine (OC) which we originally synthesized and described to selectively inhibit the cdc2 family of CDKs (e.g. cdc2, cdk2 and cdk5) but not cdk4/cdk6 kinases.[11,12] OC has already been found to potentiate mitoxantrone-induced apoptosis,[13] to inhibit proliferation of a variety of human tumor cell lines *in vitro*,[11] and, in concert with its ability to inhibit cdc2 family of CDKs, to block the cell cycle on G_1/S and G_2/M boundaries.[14] Our studies performed on several mouse tumor models clearly demonstrated the ability of synthetic CDKIs to inhibit tumorigenesis and to induce apoptosis in tumor cells both under *in vitro* and *in vivo* conditions.

In order to determine the feasibility of a clinical application of synthetic CDKIs, we tested the effect of OC on a spontaneous dog malignant melanoma in a 6-year-old mastiff. We report here the induction of massive apoptosis and tumor regression following the *in vivo* application of the drug.

Case report

A 6-year-male 65 kg weight mastiff was diagnosed in January 1997 as having a tumor of the right maxilla. The dog was suffering from occasional local bleeding and pain during digestion. Due to osteolytic and dentinolytic activity of the tumor, two premolars were extracted and a bioptic examination of the surrounding tissue confirmed the diagnosis of malignant melanoma; this disease has a poor prognosis in both the human and veterinary clinical practice.

One month later, the breeders asked our research group to treat the dog experimentally. Following consultation with our ethical committee, we agreed on an experimental treatment of the animal with OC. The clinical examination of the dog before therapy showed extensive tumor infiltration of the entire right maxilla (Figure 1), with osteolytic infiltration of the maxillary and nasal cavities, while the orbital cavity remained intact. Regional right cervical lymph nodes were heavily affected with the metastatic process. Initial laboratory examinations showed only moderate leukocytosis with increased levels of immature neutrophil elements (Table 1).

OC was synthesized as described earlier.[15] The dog was treated with the drug solubilized in 50 mM HCl–saline at a final concentration of 5 mg/ml. The OC solution was filter sterilized and applied i.v. as a 45 min infusion at a total dose of 8 mg/kg/day. The same treatment was repeated for 7 days in succession. The dosage was adopted from the schedule which showed maximum efficacy in the model of mouse B16 melanoma (Hajdúch *et al.*, submitted). However, in the course and after completion of the treatment, the tumor became necrotic and this process was accompanied by repeated bleeding from tumor tissue. At the same time as bleeding periods, there were falls in hemoglobin levels and red blood cells counts. Nevertheless, these levels recovered quickly due to the rapid regenerating capacity of dog bone marrow (Table 1). The clinical symptoms of drug toxicity were not detected, the dog being in good general condition. After the third day of OC medication, there was an increase in serum lactate dehydrogenase (LDH),

Figure 1. Photography of a dog oral cavity showing infiltration of the right maxilla and hard palate with a primary tumor.

Table 1. The course of biochemical and hematological parameters during after the OC treatment (bold columns)

	Days of treatment							
	0	1	3	5	7	10	14	21
LDH (ncat/l)	131	**125**	**318**	**432**	**527**	659	548	274
AST (ncat/l)	374	**380**	**673**	**1290**	**1763**	1347	889	660
ALT (ncat/l)	428	**415**	**519**	**487**	**453**	507	538	413
γGMT (μcat/l)	1.82	**1.93**	**1.77**	**2.05**	**1.86**	1.91	1.79	1.82
ALP (μcat/l)	2.76	**2.84**	**3.93**	**7.54**	**8.86**	6.82	5.20	4.62
Total bilirubin (μM)	17.7	**18.1**	**17.4**	**19.3**	**18.7**	20.2	19.0	18.6
Conjugated bilirubin (μM)	4.9	**5.2**	**5.6**	**6.2**	**6.3**	5.6	5.9	5.4
Hemoglobin (g/l)	178.7	**208.0**	**114.3**	**184.6**	**194.8**	153.3	197.1	201.5
Red blood cells (10^6/ml))	3.27	**2.73**	**2.32**	**3.02**	**3.17**	2.92	3.22	3.10
White blood cells (10^3/ml)	27.2	**22.6**	**21.8**	**24.0**	**22.5**	20.1	18.4	17.9
Platelets (10^3/ml)	215	**230**	**192**	**184**	**217**	205	219	223
Segments (%)	59	**55**	**60**	**58**	**57**	53	52	50
Rods (%)	22	**20**	**13**	**11**	**13**	9	8	9
Eosinophils (%)	6	**9**	**7**	**4**	**5**	8	6	5
Monocytes (%)	13	**9**	**12**	**14**	**11**	16	15	11
Lymphocytes (%)	6	**7**	**8**	**13**	**14**	14	19	25

aspartate aminotransferase (AST) and alkaline phosphatase (ALP), whilst the levels of γ-glutamyltranspeptidase (γGMT), alanine aminotransferase (ALT), and total and conjugated bilirubin remained unchanged (Table 1). Increased levels of serum LDH AST and ALP correlated with the anti-cancer properties of OC, as in the same period, apoptosis of tumor cells was detected and the normal levels of γGMT, ALT, total/conjugated bilirubin argue against organ toxicity of the drug. In the course of the treatment, repetitive fine needle biopsies from metastatic lymph nodes were performed. As early as the third day of therapy, the biopsy showed thousands of apoptotic bodies and the sporadic presence of poorly differentiated melanoma cells with only occasional melanosomes. Moreover, on the seventh day of the treatment, aspirated tissue also contained sporadic neutrophils and eosinophils. On the 10th day after completion of OC administration, fine needle biopsy showed marked infiltration of the tissue with macrophages containing ingested apoptotic bodies. However, a remaining population of tumor cells was also detected. The cytological changes in lymph nodes were accompanied by regression of metastases from 11 × 6 to 7 × 5 cm (length × width) 2 weeks after therapy. Although regression of primary tumor was not clearly observed, the tissue became necrotic, disintegrated and hemorrhaged.

In order to remove the necrotizing tumor and to prevent extensive hemorrhage attacks, surgical extirpation of tumor was recommended 2 weeks after completion of therapy. In the course of surgery, primary tumor and cervical metastases were extirpated completely. The extent of tumor infiltration corresponded to what had been expected from the clinical examination before surgery. The total weight of the removed tumor tissue was 1.98 kg. Unfortunately, the dog died soon after surgery because of respiratory depression, a common cause of death in mastiffs after long-lasting surgery. The removed tumor tissue was further analyzed by histology, electron microscopy and tested for *in vitro* drug resistance by 3-/4,5-dimethylthiazol-2,5-diphenyl/tetrazolium bromide (MTT) assay.

A histopathological examination of tissue samples before the treatment revealed nets of tumor cells surrounded by a fine fibrous stroma (Figure 2a). Some cells showed melanine production (Figure 2b). Vascular obturations and areas of minimal necrosis (17%) were occasionally observed in the central part of the untreated tumor. Apoptotic and mitotic indices showing the percentage of apoptotic/mitotic cells in the tumor were 0.08 and 2.1%, respectively. Histology was consistent with diagnosis of malignant melanoma. Two weeks after completion of the OC treatment, the area of central necrosis was extremely extended (68.2%), with only minimal neutrophil infiltration thoughout (Figure 2c). Tumor cells exhibited typical nuclear disintegration and numerous apoptotic bodies were observed (Figure 2d). The percentage of apoptotic cells in non-necrotic areas increased to 2.23% and mitotic index was 1.74%. Focally, phagocytosis of cellular and nuclear fragments by macrophages was demonstrated. In addition to the morphological markers of programmed cell death, specific *in situ* labeling of DNA breaks in apoptotic cells was performed by terminal deoxynucleotidetransferase

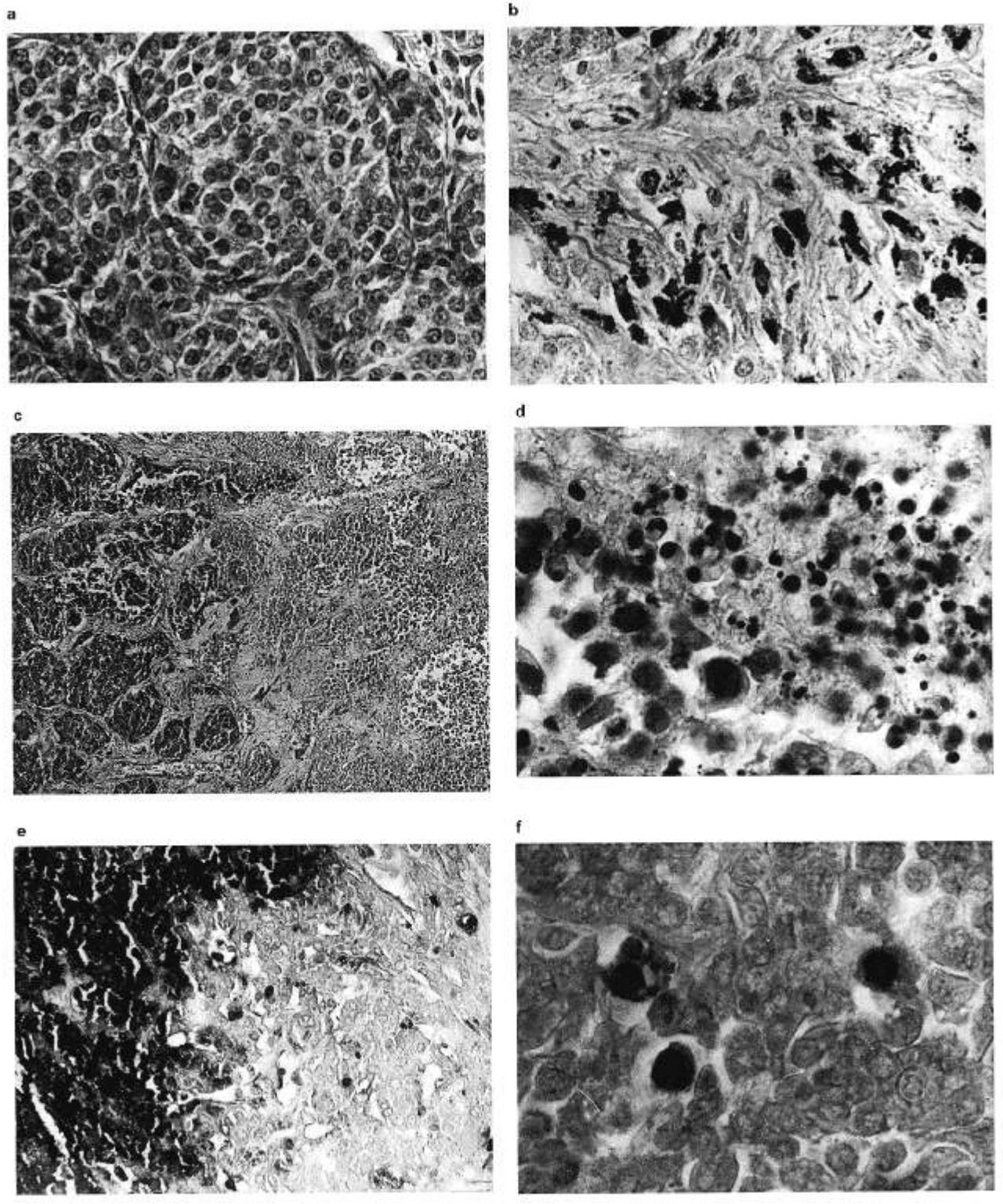

Figure 2. Photomicrographs of hematoxylin & eosin-stained and TUNEL-labeled tissue samples of dog malignant melanoma. (a) Typical histological structure of untreated tumor (×110). (b) Production of melanine by certain tumor cells (×220). (c) Extensive secondary necrosis in OC-treated tumor (×25). (d) Detail of (c) (×300) demonstrates numerous typically disintegrated nuclei (karyorhexis) without granulocyte infiltration, occasionally with the macrophage-mediated phagocytosis of apoptotic bodies. (e) Intensive positivity of secondary necrotizing areas of treated tumor by TUNEL (×90), while single TUNEL-labeled cells were located on the periphery of necrotic zones. (f) Detail of (e) TUNEL-positive nuclei of melanoma cells (×300).

nick end-labeling assay (TUNEL) according to the protocol described by Gavrieli *et al.*[16] (In Situ Cell Death Detection Kit/AP; Boehringer, Mannheim, Germany). The positivity of the TUNEL assay supported an apoptotic mode of cell death induced by OC (Figure 2E and F).

Ultrastructural examination of the extirpated tumor following OC treatment was performed on glutaraldehyde-fixed, osmium tetraoxide-postfixed, epoxyde polymerized ultra-thin sections of the tissue contrasted with uranium acetate. Melanoma cells with melanosomes and premelanosomes in the cytoplasm (Figure 3a and 3b) were located within the extracellular matrix, mostly formed by secondary necrotizing apoptotic malignant cells. However, the majority of tumor cells were heavily apoptotic with numbers of fragmented nuclei containing condensed chromatin (Figure 3C). The formed apoptotic bodies were rapidly phagocytosed by tissue macrophages (Figure 3d).

The evaluation of the *in vitro* drug sensitivity assay was performed on a suspension of vital melanoma cells isolated from the primary tumor according to Hajdúch *et al.*[17] Cells were cultured *in vitro* with increasing amounts of drugs. Tumor cell survival was evaluated by the MTT assay and the concentration killing 50% of tumor cells (TCS_{50}) was calculated as

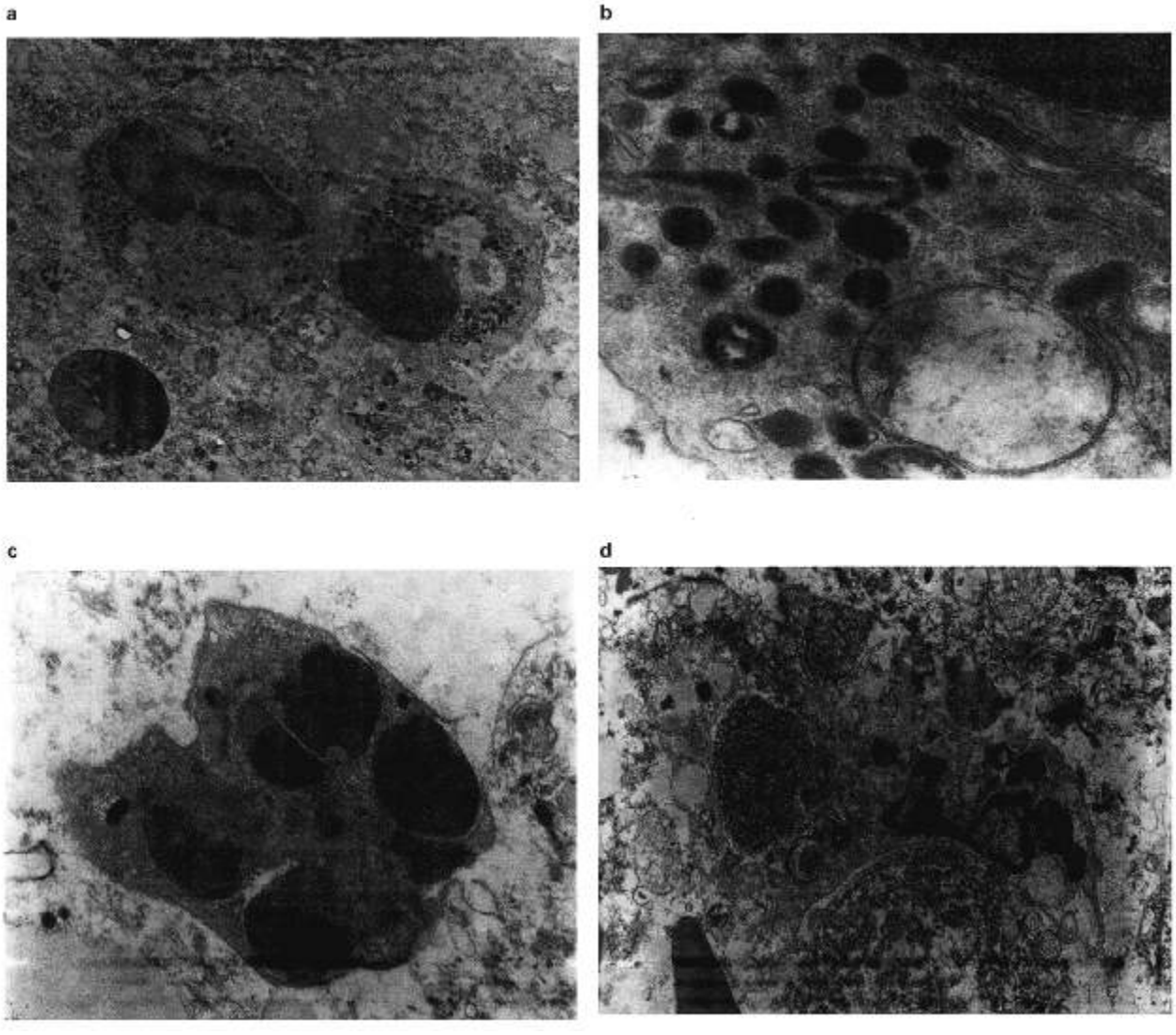

Figure 3. Electron microscopy photographs of dog malignant melanoma following OC treatment. (a) Two melanoma cells lacking apparent morphological markers of apoptosis (×6354). (b) Detail of (a) showing formation of melanosomes and premelanosomes in the cytoplasm of tumor cells is compatible with diagnosis of malignant melanoma (×30650). (c) Heavily apoptotic melanoma cell exhibiting chromatin condensation and nuclear fragmentation (×18682). (d) Tissue macrophage phagocytosing apoptotic bodies in the tumor (×6130).

Table 2. Results of drug sensitivity MTT assay of melanoma cells isolated from the dog following OC treatment: chemosensitivity of mouse melanoma cell line B16 and cancer cells of a typical patient suffering from malignant melanoma are entered for comparison.

	Cytotoxicity (TCS_{50})		
	Dog melanoma	Mouse B16 melanoma	Human melanoma
Olomoucine (μM)	32.35	42.15	82.30
Daunorubicin (μg/ml)	0.30	0.29	0.12
Vincristin (μg/ml)	>50.00	>50.00	>50.00
Vepesid (μg/ml)	>50.00	>50.00	>50.00
Taxol (μg/ml)	14.34	20.16	31.49
Actinomycin D (μg/ml)	<0.02	0.05	<0.02
Cisplatin (μg/ml)	14.30	17.52	2.26
Mitoxantron (μg/ml)	>2.00	>2.00	>2.00

[a]50% tumor cell survival, the concentration of drug killing 50% of tumor cells.

described elsewhere.[17] The results of the assay demonstrated the resistance of the melanoma cells to most traditional anti-cancer drugs, while the level of tumor cell sensitivity to OC was comparable to many unrelated cell lines tested in our laboratory (Hajdúch *et al.*, unpublished results). Analogous results were obtained when performing tests on drug resistance of tumor cells isolated from mouse B16 melanoma or spontaneous human malignant melanoma. This observation could indicate that OC and other synthetic CDKI are not transported/inactivated by mechanisms of multidrug resistance.

Discussion

It is reported here that the inhibition of the cdc2 family of CDKs by the synthetic CDKI OC resulted in initiating programmed cell death in a case of dog malignant melanoma. This observation is consistent with the ability of OC to potentiate mitoxantrone-induced cytotoxicity,[13] and with the ability of other, more effective, synthetic CDKIs recently prepared in our laboratories to initiate massive apoptosis in tumor cells (Hajdúch *et al.*, submitted). Although the exact mechanism of OC triggered apoptosis is still unclear, we may hypothesize that down-regulation of crucial CDKs on G_1, G_2 and M cell cycle check points is incompatible with further survival of the actively proliferating cancer cells.

The resistance of p53-deficient tumors to DNA damaging therapy and radiation has already been demonstrated in clinical practice.[18,21] In this respect, the application of a synthetic CDKI like OC could partially substitute the p53-deficient pathway in tumor cells and definitely induce apoptosis, differentiation and in some cases restore susceptibility of cancer cells to DNA damaging drugs and radiation. The significance of this capacity is further supported by the fact that mutations in the p53 gene belong to the most frequent alterations of cancer cells.[9]

The mechanism of OC action appears to be different from currently used anti-cancer drugs. Based on the results of the MTT assay it seems evident that OC is able to overcome the mechanisms of natural/therapy-induced drug resistance of melanoma cells. This conclusion is further supported by the fact that OC is equally effective in DNA synthesis inhibition both in original MCF-7 or KB-3-1 cells and their adriamycin-resistant sublines.[12]

In conclusion, it should be stressed that OC showed higher anti-cancer properties than initially realized for this relatively weak synthetic CDKI. It seems possible that the ability of OC and related compounds to induce apoptosis, and thus constrain tumor cell proliferation, together with its capacity to cross the mechanisms of multidrug resistance, may provide a significant therapeutic benefit for cancer patients.

Acknowledgments

The collaboration of dog owners L Ditrtová and P Ditrt is greatly appreciated. The authors are indebted to Professor R Kod'ousek for the preparation of micro-photographs and for fruitful discussion. We are grateful to Mrs Jarmila Potomková for editorial help.

References

1. Grana X, Reddy EP. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* 1995; **11:** 211-9.
2. El-Deiry WS, Harper JW, O'Connor PM, *et al.* WAF1/CIP1 is induced in p53-mediated G_1 arrest and apoptosis. *Cancer Res* 1994; **54:** 1169-74.
3. Firpo EJ, Koff A, Solomon MJ, Roberts JM. Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol Cell Biol* 1994; **14:** 4889-901.
4. Lee MH, Reynisdottir I, Massague J. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* 1995; **9:** 639-49.
5. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993; **366:** 634.
6. Reynisdottir I, Polyak K, Iavarone A, Massague J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle

arrest in response do TGF-beta. *Genes Dev* 1995; **9:** 1831-45.
7. Guan KL, Jenkins CW, Li Y, *et al.* Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev* 1994; **8:** 2939-52.
8. Chan FK, Zhang J, Cheng L, Shapiro DN, Winoto A. Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. *Mol Cell Biol* 1995; **15:** 2682-8.
9. Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. *Nature* 1991; **351:** 453-6.
10. Yang ZY, Perkins ND, Ohno T, Nabel EG, Nabel GJ. The p21 cyclin-dependent kinase inhibitor suppress tumorigenicity *in vivo*. *Nat Med* 1995; **1:** 1052-6.
11. Vesely J, Havlicek L, Strnad M, *et al.* Inhibition of cyclin-dependent kinases by purine derivates. *Eur J Biochem* 1994; **224:** 771-86.
12. Schulze-Gahmen U, Brandsen J, Jones HD, *et al.* Multiple modes of ligand recognition: crystal structures of cyclin-dependent protein kinase 2 in complex with ATP and two inhibitors, olomoucine and isopentenyladenine. *Proteins* 1995; **22:** 378-91.
13. Ongkeko W, Ferguson DJP, Harris AL, Norbury C. Inactivation of Cdc2 increases the level of apoptosis induced by DNA damage. *J Cell Sci* 1995; **108:** 2897-904.
14. Abraham RT, Acquarone M, Andersen A, *et al.* Cellular effects of olomoucine, an inhibitor of cyclin-dependent kinases. *Biol Cell* 1995; **83:** 105-20.
15. Havlicek L, Hanus J, Vesely J, *et al.* Cytokinin-derived cyclin-dependent kinase inhibitors: synthesis and cdc2 inhibitory activity of olomoucine and related compounds. *J Med Chem* 1997; **40:** 408-12.
16. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J Cell Biol* 1992; **119:** 493-501.
17. Hajduch M, Mihal V, Minarik J, *et al.* Decreased *in vitro* chemosensitivity of tumor cells in patients suffering from malignant diseases with a poor prognosis. *Cytotechnology* 1996; **19:** 243-5.
18. Benhattar J, Cerottini JP, Saraga E, Mettthez G, Givel JC. p53 mutations as a possible predictor of response to chemotherapy in metastatic colorectal carcinomas. *Int J Cancer* 1996; **69:** 190-2.
19. Aas T, Borresen AL, Geisler S, *et al.* Specific p53 mutations are associated with *de novo* resistance to doxorubicin in breast cancer. *Nat Med* 1996; **2:** 811-4.
20. Newcomb EW. p53 gene mutations in lymphoid diseases and their possible relevance to drug resistance. *Leuk Lymph* 1995; **17:** 211-21.
21. Muro K, Ohtsu A, Boku N, *et al.* Association of p53 protein expression with responses and survival of patient with locally advanced esophageal carcinoma treated with chemoradiotherapy. *Jpn J Clin Oncol* 1996; **26:** 65-9.

(Received 3 July 1997; revised form accepted 28 August 1997)