Abstract. Background/Aim: Constitutive activation of nuclear factor kappa-B (NFκB) is a hallmark of various cancer types, including melanoma. Chemotherapy may further increase tumour NFκB activity, a phenomenon that, in turn, exacerbates drug resistance. This study aimed at preliminary screening of a panel of aromatic aldehydes, including vanillin, for cytotoxicity and suppression of tumour cell NFκB activity. Materials and Methods: The cytotoxic and NFκB-inhibitory effects of 10 aromatic aldehydes, including vanillin, were investigated in cultured A375 human melanoma cells. Each compound was assayed alone and in combination with the model NFκB-activating drug doxorubicin. The most promising analogues were then tested alone and in combination with 4-hydroperoxycyclophosphamide in vitro, and with cyclophosphamide in mice bearing A375 xenografts. Results: The vanillin analogues o-vanillin and 2,4,6-trihydroxybenzaldehyde exhibited cytotoxicity against cultured A375 human melanoma cells. Each compound was assayed alone and in combination with the model NFκB-activating drug doxorubicin. The most promising analogues were then tested alone and in combination with 4-hydroperoxycyclophosphamide in vitro, and with cyclophosphamide in mice bearing A375 xenografts. Results: The vanillin analogues o-vanillin and 2,4,6-trihydroxybenzaldehyde exhibited cytotoxicity against cultured A375 cells, and inhibited doxorubicin- and 4-hydroperoxycyclophosphamide-induced NFκB activation. They also suppressed A375 cell growth in mice. Conclusion: o-vanillin and 2,4,6-trihydroxybenzaldehyde deserve further evaluation as potential anticancer drugs.

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Nuclear factor kappa-B (NFκB) is a transcription factor playing a crucial role in malignant diseases (1-4). Up-regulation of NFκB activity is detected in various human tumours (5-7), including pancreatic adenocarcinoma (8), breast cancer (9), and melanoma (10), where it may contribute to malignant behaviour. In particular, NFκB activation has been associated with cancer development and progression (5, 11), and may inhibit apoptosis and favour cancer cell proliferation, invasion, angiogenesis, and metastasis (12, 13). Importantly, increased NFκB activation may induce tumour immune escape and chemotherapy resistance (14-16). Chemotherapy-induced cellular stress, in turn, might further increase NFκB activity of tumour cells, protecting them from chemotherapy-induced apoptosis (17-19). Therefore, NFκB signaling pathways could serve as potential targets for cancer therapy.

Vanillin (4-hydroxy-3-methoxybenzaldehyde; compound 10, Table I) is a major component of the bean and pod of some plant species of the Vanilla genus, and is also synthesized on a large scale for use as a flavouring agent in food, fragrance and pharmaceutical industries. Multiple biological effects have been documented for vanillin. It exhibits antioxidant (20, 21), antimicrobial (22), analgesic (23, 24) and anti-sickling (25) properties. It was also proven to be an anticarcinogen in rats (26) and an antimutagen in a variety of in vitro models (27-29). Vanillin is relatively non-cytotoxic towards cultured mammalian cells, but does potentiate the cytotoxicity of some DNA-damaging agents, including cisplatin (30) and mitomycin C (31), a property which correlates with its ability to impair DNA double-strand break repair via inhibition of DNA protein kinase (30). Although unable to suppress primary tumour growth itself, vanillin was found to exert anti-metastatic activity in the 4T1 mouse mammary carcinoma spontaneous metastasis model, and to inhibit tumour cell invasion and migration in vitro.
inactivated foetal bovine serum (FBS; Lonza). Cultures were grown supplemented with 10% heat-inactivated Eagle's medium/nutrient mixture F-12 (DMEM/F12) medium (Manassas, VA, USA) and maintained in Dulbecco’s modified immediately before use.

−20˚C. 4-HC and cyclophosphamide were dissolved in sterile PBS at 10 mM was prepared in sterile sodium chloride 0.9% and kept at sterile phosphate-buffer saline (PBS). A stock solution of doxorubicin for the discovery of more effective analogues.

In this article, we report the results of in vitro experiments undertaken to screen a small set of structurally related aromatic aldehydes, including vanillin, for cytotoxic activity, and for inhibition of constitutive and chemotherapy-induced NFκB activity in human melanoma cells. Furthermore, we report the results of a trial in A375 human melanoma-bearing mice evaluating the efficacy of two selected aldehydes, namely o-vanillin and 2,4,6-trihydroxybenzaldehyde, as single agents and in combination with the NFκB-inducing drug cyclophosphamide.

Materials and Methods

Chemicals and working solutions. The anticancer drugs doxorubicin (as hydrochloride salt) and cyclophosphamide, as well as all the aromatic aldehydes used throughout the study, including vanillin (4-hydroxy-3-methoxybenzaldehyde; compound 10), the vanillin isomer o-vanillin (2-hydroxy-3-methoxybenzaldehyde; compound 7), and 2,4,6-trihydroxybenzaldehyde (compound 6) (see Table I), were obtained from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroperoxycyclophosphamide (4-HC) was obtained from Nissleh-IIT GmbH (Bielefeld, Germany). Stock solutions of each aldehyde at 100 mM were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich), stored at 4˚C, and used within 6 weeks. Working solutions were prepared just before use by dilution of the stocks with cell culture medium (see below); the final concentration of DMSO in the culture medium never exceeded 0.25% (v/v). Suspensions for oral attach for 24 h. They were then grown (37˚C; 5% CO 2) for an additional 48 h in the absence (control) or in the presence of 250 μM of the tested aromatic aldehyde, or in the presence of increasing concentrations of doxorubicin (0.625-10 μM) or 4-HC (3.125-50 μM). Cell viability was then assessed using an XTT colorimetric assay (Cell Proliferation Kit XTT; AppliChem, Darmstadt, Germany) that measures the activity of dehydrogenase enzymes of viable cells (37). Within each experiment, determinations were performed in duplicate or triplicate, and experiments were repeated at least five times. The percentage cell viability was defined as the absorbance of the drug-treated wells expressed as a percentage of that of controls.

Animals. Twelve- to sixteen-week-old male NSG mice (NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ) were purchased from Charles River Hungary Ltd. (Budapest, Hungary). They were maintained in the specific pathogen-free animal facility of the Biological Research Center (BRC, Szeged, Hungary). Six- to eight-week-old inbred male Balb/c mice were obtained from the animal facility of the BRC. They received commercial mouse food pellets and water ad libitum.
Table I. Cytotoxic and nuclear factor kappa-B-inhibitory effects of different aromatic aldehydes in A375 human melanoma cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Cell viability (% of control)</th>
<th>NFκB activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aldehyde alone (250 μM)</td>
<td>Doxorubicin (1.25 μM) + aldehyde (250 μM)</td>
</tr>
<tr>
<td>1 2,4,6-Trimethoxybenzaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>44</td>
<td>11</td>
</tr>
<tr>
<td>2 2,5-Dimethoxybenzaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>50</td>
<td>21</td>
</tr>
<tr>
<td>3 2,4-Dihydroxybenzaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>83</td>
<td>25</td>
</tr>
<tr>
<td>4 4-Nitrobenzaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>84</td>
<td>22</td>
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<tr>
<td>5 2-Nitrobenzaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>38</td>
<td>14</td>
</tr>
<tr>
<td>6 2,4,6-Trihydroxybenzaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>7 2-Hydroxy-3-methoxybenzaldehyde (α-Vanillin)</td>
<td><img src="image" alt="Structure" /></td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>8 4-(Dimethylamino)benzaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>104</td>
<td>36</td>
</tr>
<tr>
<td>9 3-Quinolinecarboxaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>77</td>
<td>30</td>
</tr>
<tr>
<td>10 4-Hydroxy-3-methoxybenzaldehyde (Vanillin)</td>
<td><img src="image" alt="Structure" /></td>
<td>104</td>
<td>26</td>
</tr>
</tbody>
</table>

*Cell viability was evaluated after continuous 48-h exposure to aldehyde or aldehyde plus doxorubicin. Data reported are means of 3-5 experiments.
†Luciferase activity was evaluated after a 6 h-exposure time. Data reported are means of 3-5 determinations. *Doxorubicin concentration was 1.25 μM; ‡exposure to doxorubicin (1.25 μM) alone resulted in 15% cell viability.
Explorative toxicology study. The in vivo antitumor activity trial was preceded by an explorative multiple-dose toxicology study carried out in healthy male Balb/c. Animals were randomly assigned to each experimental group (n=3/group) and were treated once a day orally with o-vanillin (60 mg/kg), TBA (60 mg/kg), or PBS (control) for 5 consecutive days followed by a 2-day wash-out. Mice were examined daily for a decrease in physical activity, weight loss, and other signs of disease. After 3 weeks of treatment, the animals were sacrificed and major organs (intestine, liver, kidneys, spleen, heart, and lungs) were visually inspected for any abnormalities. Treatment-related signs of toxicity were not observed, neither during the 14-day treatment period, nor at macroscopic post-mortem examination.

A375 xenograft and treatment. NSG mice were subcutaneously injected with 2×10^6 A375 cells suspended in 100 μl of RPMI 1640 (Lonza). One day later, the animals were randomly assigned to the following experimental groups (n=5-8/group): a: o-vanillin (60 mg/kg); b: cyclophosphamide (80 mg/kg); c: o-vanillin (60 mg/kg) plus cyclophosphamide (80 mg/kg); d: TBA (60 mg/kg); c: TBA (60 mg/kg) plus cyclophosphamide (80 mg/kg); f: control group. The aldehydes were administered once a day orally for 5 consecutive days followed by a 2-day wash-out, starting 2 days after tumour implantation. The aldehydes were administered intraperitoneally (i.p.) on day 7 and 14 after tumour cell injection; the dose and schedule of cyclophosphamide were chosen on the basis of previous optimal dose/schedule-finding experiments, which had been carried out in mice bearing 4T1 mouse mammary carcinomas (Vizler et al., unpublished data). Tumour volume was estimated from measurements made with a calliper using the formula: tumour volume (mm^3)=D × d^2; where D and d values were the longest and the shortest diameters of the tumour, respectively. For ethical reasons, all the animals were sacrificed at day 20.

Results

Selected vanillin analogues remarkably inhibit NFκB activation and A375 human melanoma cell growth in vitro. Two widely used anticancer drugs capable of inducing NFκB activity are doxorubicin and cyclophosphamide (38). A set of experiments analyzed the impact of doxorubicin on NFκB activity in A375 human melanoma cells stably transfected with an NFκB-luciferase reporter construct (A375/NFκB-Luc cells). The dotted line represents the NFκB activity of untreated cells (basal NFκB activity). A: Doxorubicin (1.25 μM) caused a more than 3-fold increase of NFκB activity compared to control (p<0.0001). B: o-vanillin (compound 7; final concentration: 250 μM) significantly reduced basal NFκB activity of A375/NFκB-Luc cells (p<0.0001 vs. control). On the contrary, vanillin (compound 10; final concentration: 250 μM) and TBA (compound 6; final concentration: 250 μM) did not significantly affect basal NFκB activity of A375/NFκB-Luc cells. C: Induction of NFκB activity by 1.25 μM doxorubicin was significantly suppressed by 250 μM 2,4,6-trihydroxibenzaldehyde (compound 6; p=0.0020 vs. doxorubicin alone) or 250 μM o-vanillin (p<0.0001 vs. doxorubicin alone). Values are mean±SD (n=3). Statistical comparison was performed by unpaired, two-tailed t-test.
time. As shown in Figure 1A, the maximum inductive effect of doxorubicin was observed at 1.25 μM, resulting in a more than 3-fold increase in luciferase activity over the basal level. This doxorubicin concentration was, therefore, selected for the subsequent assays. Six out of the 10 tested aldehydes (namely compounds 1, 3, 5, 6, 7 and 9) but not vanillin (compound 10) were effective in inhibiting the induction of NFκB transcriptional activity by doxorubicin by more than 20%. Results of these experiments are summarized in the last column of Table I. Interestingly, a vanillin isomer, o-vanillin (compound 7), was the most potent compound, inhibiting doxorubicin-mediated induction of NFκB activity by 65% (Figure 1C). This aldehyde was also active in suppressing constitutive (basal) NFκB activity in A375 cells (Figure 1B).

The inhibitory effect of o-vanillin (compound 7) on chemotherapy-induced NFκB activity in A375 human melanoma cells was confirmed using 4-hydroperoxycyclophosphamide (4-HC), an in vitro analogue of the produg cyclophosphamide, as the NFκB activator. The maximum induction of luciferase activity by 4-HC occurred at 12.5 μM, where 250 μM o-vanillin suppressed 4-HC-induced activity by 43% (Figure 2). Based on the results of the in vitro tumour cell growth-inhibition assay (see below), a second benzaldehyde derivative, namely TBA (compound 6), was evaluated for its ability to counteract NFκB induction by 4-HC; 250 μM TBA inhibited 4-HC-elicited induction of NFκB by 17% (data not shown).

The same aromatic aldehydes were further examined in vitro at a fixed concentration (250 μM) for cytotoxicity as single agents and in combination with the NFκB-activating anticancer drug doxorubicin; cell viability was evaluated using an XTT assay after a continuous 48-h exposure to the studied compounds. Results of these studies are summarized in Table I. Among the tested aldehydes, only TBA and o-vanillin exhibited remarkable cytotoxicity when tested alone, reducing A375 cell viability to 10% or less when combined with 1.25 μM doxorubicin (Table I).

**o-Vanillin and TBA exert significant therapeutic activity in mice bearing A375 human melanoma xenografts.** Based on the above-described in vitro findings, o-vanillin and TBA were selected for an in vivo efficacy trial in tumour-bearing mice, as single agents and in combination with cyclophosphamide; the dose and schedule of administration of the studied aldehydes were based on the results of an explorative toxicology study carried out in Balb/c mice, demonstrating no gross toxicity signs and weight loss.

As shown in Figure 3, oral administration of o-vanillin, or TBA, as a single agent or in combination with i.p. cyclophosphamide, delayed the growth of A375 human melanoma xenografts in immunodeficient NSG mice (see legend of Figure 3 for details on the doses and schedules of administration of each agent). It is worth noting that the growth-delaying effect of the combination o-vanillin/ cyclophosphamide reached statistical significance by day 15, and remained statistically significant until the end of the experiment (i.e. day 20). Moreover, on day 20, the antitumor effect of both tested aldehydes, as single agents, was strongly significant; the growth inhibition was 45% in the case of TBA and 32% in the case of o-vanillin, respectively (p<0.0001 vs. control). Importantly, it was comparable to that exerted by the established anticancer drug cyclophosphamide. Finally, the statistical analysis showed that on day 20, the combination of o-vanillin and cyclophosphamide was more effective than o-vanillin alone (p<0.05).

**Discussion**

NFκB signaling pathways are constitutively active in many tumour types and have been implicated in cancer cell proliferation, invasion, metastasis and angiogenesis, as well as in suppression of cancer cell apoptosis (12). Moreover, some anticancer agents may induce chemoresistance of cancer cells through activation of NFκB (14, 16, 17). Therefore, NFκB is currently considered an ideal target for cancer therapy (2), and various NFκB inhibitors targeting different components of NFκB activation, that is, inhibitor of kappa B kinase or NFκB subunits, are under development (39).

The observation that the widely-used natural flavouring agent vanillin (4-hydroxy-3-methoxybenzaldehyde; compound 10, Table I) behaves as an NFκB inhibitor in both in vitro and in vivo preclinical models (34-36) led us to explore the ability.
of a small panel of aromatic aldehydes, including vanillin, to suppress chemotherapy-induced NFκB activity, and to inhibit tumour cell growth. Even though, surprisingly, vanillin was ineffective in modulating NFκB activity under our experimental conditions, six out of the 10 tested aldehydes (namely compounds 1, 3, 5, 6, 7 and 9) were effective in suppressing NFκB induction by the anticancer drug doxorubicin in cultured A375 human melanoma cells (Table I), with o-vanillin, a naturally occurring isomer of vanillin (40), being the most potent compound (Table I, and Figure 1C). The remarkable anti-NFκB activity exhibited by o-vanillin as well as its significant tumour growth-inhibiting activity both in vitro (Table I) and after oral administration at a non-toxic schedule to mice bearing A375 human melanoma xenografts (Figure 3) suggest that this vanillin analogue deserves further preclinical investigation as a potential antitumor drug. Although toxicological data on o-vanillin are scant, the published data of acute oral toxicity in mice (median oral lethal dose: 1330 mg/kg) (41), together with the results of our explorative multiple-dose toxicology study, make this compound promising in terms of host toxicity potential.

Besides o-vanillin, our screening highlighted the aromatic aldehyde TBA, as a compound endowed with both a moderate ability to counteract induction of NFκB activity by doxorubicin or 4-HC in A375 human melanoma cells, and a remarkable in vitro and in vivo growth-inhibitory activity towards the same cell line (Table I and Figure 3). TBA is one several metabolites of anthocyanins formed by gut microflora (42), and has been recently found to exhibit both in vitro antiproliferative activity towards human colorectal cancer cell lines, as well as an inhibitory effect on NFκB DNA-binding activity in Caco-2 cells (43).

In conclusion, a preliminary screening of a small panel of vanillin analogues for tumour growth inhibition and suppression of NFκB signalling in A375 human melanoma cells has led to the identification of o-vanillin and TBA as compounds deserving further preclinical evaluation as potential anticancer agents both alone and in combination regimens. We plan to perform systematic preclinical studies in order to further explore the anticancer potential of the selected aldehydes, as well as to clarify the role of NFκB inhibition in their antitumor activity.

Acknowledgements

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