

Inhibitory effect of vanillin-like compounds on respiration and growth of adenocarcinoma TA3 and its multiresistant variant TA3-MTX-R

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Abstract

The effects of some imine and amine derivatives of vanillin on the respiration rate of mouse mammary adenocarcinoma TA3 line, its multiresistant variant TA3-MTX-R line and mouse hepatocytes, together with their respective mitochondrial fractions, are described. These derivatives inhibit respiration in both tumour cell lines more effectively than vanillin in the absence or presence of the uncoupler CCCP. Since both types of derivatives block the electron flow, mainly through the NADH-CoQ span, they behave as oxidative phosphorylation inhibitors. Thus, they prevent ATP synthesis and alter cellular processes requiring energy, which would lead to cellular death. Amine derivatives of vanillin present a similar effect on both tumour cell lines, being amine C the most efficient inhibitor. Moreover, mouse hepatocytes are about 4-fold less sensitive to amine C than tumour cells. These amine derivatives are better inhibitors than the corresponding imines; probably because they should interact better with the respiratory chain reaction site.

Keywords: Vanillin; Amines; Imines; Mitochondria; Tumour cell lines; Oxidative phosphorylation

1. Introduction

Neoplastic transformation may affect gene expression at both nuclear and mitochondrial levels, altering several metabolic pathways and metabolite transport systems. Significant increases of protein levels implicated in the oxidative phosphorylation process are also involved (Baggetto, 1993; Cotton and Rogers, 1993; Penta et al., 2001). Thus, mitochondria isolated from tumour cells exhibit increased rates of ATP synthesis as compared with normal cells (Baggetto, 1993). Electrochemical potentials of inner mitochondrial membranes of neoplastic cells are higher than those of normal cells (Singh and Moorehead, 1992). However, respiration rates of transformed cells are significantly lower than those of normal cells, which may be due to mitochondrial dysfunction and/or loss in its content (Pedersen, 1978; Wilkie, 1979). Many tumour cells show a decreased mitochondrial mass of about 50%; however, the

precise relationships between mitochondrial mass, the level of mitochondrial mRNA and the mtDNA copy number have yet to be examined. Since the mitochondrial mass of fetal rat liver is about half that of an adult, growth and reduced mitochondrial mass may be related (Penta et al., 2001). The metabolism of fast-growth and largely de-differentiated tumour cells differs markedly from that of the original tissue cells. Fast oxidation of glucose and the active production of lactic acid are some of the main properties of these cells (Pedersen, 1978; Baggetto, 1993). Glucose consumption is not the only fast catabolic pathway: glutamine and ketone bodies also appear to be rapidly oxidized. It has recently been estimated that cellular ATP is mainly provided by oxidative phosphorylation and that the NADH-CoQ span of the respiratory chain (energy-conserving site I) exerts significant control over oxidative phosphorylation, particularly in the presence of glucose, in AS-30D hepatoma cells (Rodríguez-Enríquez et al., 2000).

These changes in the oxidative phosphorylation system of tumour cells offer a useful pharmacological strategy for development of selective agents. Thus, relatively low doses

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of rotenone promote a strong growth inhibition and apoptosis in HL-60 leukemia cells (Matsunaga et al., 1996).

At present, drugs used in cancer treatment show significantly different effects on malignant cells, especially in development of resistance (Rang and Dale, 1991). Some results indicate that various plant constituents exhibit anticancer roles. Phenolic compounds are well-known antineoplastic agents, probably related to the onset of carcinogenesis (Hogman, 1989; Wattenberg, 1992; Tsuda et al., 1994). We have previously reported that mono or polyphenols inhibit respiration of tumour cells, mainly by blocking electron flow through the respiratory chain at the energy-conserving site 1 level (Fones et al., 1989; Pavani et al., 1994).

Vanillin, 3-methoxy-4-hydroxybenzaldehyde, is extracted from vanilla, potato skin, aromatic resins and oriental medicinal plants (Hogman, 1989; Wilkie, 1979). It prevents initiation of hepatocarcinogenesis induced by 2-amino-3-methylimidazole and quinoline (Akagi et al., 1995; Sanyal et al., 1997; Tsuda et al., 1994), and it appears to inhibit carcinogenesis by attenuating mutagenic effects of some heterocyclic amines (Fahrig, 1996). However, other studies have not shown these effects (Sawa et al., 1999).

These evidences encouraged us to synthesize four vanillin derivatives, and to assess their effects on the respiratory rate and cytotoxicity in the mouse mammary adenocarcinoma TA3 and its multiresistant derivative TA3-MTX-R cell lines (Morello et al., 1995)

2. Experimental procedures

2.1. Materials

Vanillin, sodium borohydride, and sodium bicarbonate were purchased from E. Merck. Albumin (fatty acid free), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), collagenase (for hepatocytes isolation), ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), glutamine, duroquinone, rotenone, *N,N,N',N'*-tetramethyl-*p*-phenyldiamine (TMPD), fetal bovine serum, and Dulbecco's modified Eagle's medium were from Sigma. Methotrexate (MTX) was from OncoPharma. All other reagents were of the highest grade available. Synthesized derivatives were dissolved in ethanol or dimethyl sulfoxide (DMSO). There were no effects of these solvents on cell growth or oxygen consumption at concentrations used.

2.2. Syntheses of vanillin derivatives

Imines were synthesized by vanillin condensation with *p*-toluidine or aniline. The respective amines were prepared by reduction with sodium borohydride (Fig. 1). Melting point determinations and thin-layer chromatog-

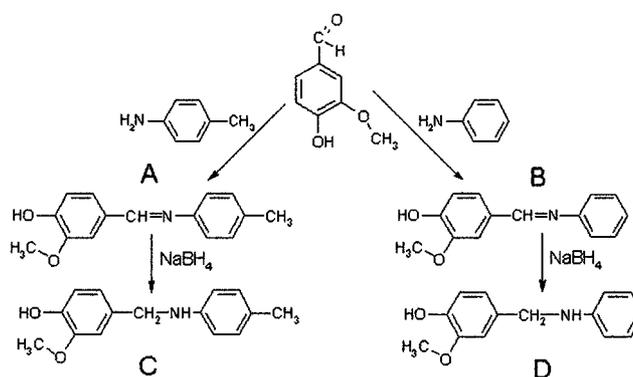


Fig. 1. Scheme of syntheses of vanillin-like compounds. (A) *N*-(3-methoxy-4-hydroxybenzylidene)-*p*-toluidine; (B) *N*-(3-methoxy-4-hydroxybenzylidene)-aniline; (C) *N*-(3-methoxy-4-hydroxybenzyl)-*p*-toluidine; (D) *N*-(3-methoxy-4-hydroxybenzyl)-aniline.

raphy were the purity tests. Characterization was by quantitative elemental analyses of C, H, and N (error $\pm 0.5\%$) and infrared and ¹H NMR spectroscopy.

2.3. Harvesting tumour cells

TA3 ascites tumour cell line was grown by weekly intraperitoneal injection of 1.0×10^6 cells into young adult male CAF 1 Jax mice. The methotrexate-resistant cell line (TA3-MTX-R) was generated by weekly consecutive selection in the presence of MTX, where 2.0×10^6 cells were propagated in mice and MTX was administered by the i.p. route, starting at 0.1 mg MTX per kg body weight every 48 h with stepwise increases to 1.0 mg/kg per 48 h up to the day of assay (Morello et al., 1995). All animals were fed with a standard laboratory chow and water ad lib. Tumour cells were harvested 5–7 days after intraperitoneal inoculation of ascites fluid from donor mice by centrifugation at $100 \times g$ for 2 min at 4 °C, and washed twice with 150 mM NaCl, 5 mM KCl and 10 mM Tris-HCl, pH 7.4, as described by Moreadith and Fiskum (1984). Tumour cells were resuspended in the same medium at the concentration of 30–40 mg protein per ml. The cells appeared to be virtually free from erythrocytes and other contaminants. Protein concentrations were determined by the Lowry reaction and standardized with serum albumin (Lowry et al., 1951).

2.4. Isolation of mouse hepatocytes

This was carried out by circulating collagenase perfusion of livers, according to a modified procedure (Moldeus et al., 1978). Livers were first perfused in situ with the oxygenated washing solution (95% O₂ and 5% CO₂) containing calcium-free salt and 0.5 mM EGTA (10 ml/min at 37 °C for 5 min), followed by perfusion with a solution containing 0.04% collagenase for 10 min. The liver was then gently minced on a Petri dish and filtered

with nylon mesh. Hepatocytes were washed twice with 150 mM NaCl, 5 mM KCl and 10 mM Tris-HCl, pH 7.4, and centrifuged at $50\times g$ for 2 min. They were then resuspended in the same medium at 30–40 mg protein/ml concentration. Cell viability was consistently >90% as determined by trypan blue exclusion.

2.5. Cell respiration

Respiration measurements were carried out polarographically at 25 °C with a Clark electrode (Yellow Springs Instrument) and a Linseis L 4000 monitor linked to a 200 mV monochannel recorder. The reaction mixture, 1.8 ml, contained 150 mM NaCl, 5 mM KCl, and 10 mM Tris-HCl, pH 7.4, plus 5.6 mM glutamine (tumour cells) or 10 mM glucose plus 5 mM glutamate (hepatocytes) or 5 mM succinate as respiratory substrates and 5 mg of protein of either ascites tumour cells (about 10^7 cells) or hepatocytes (about 6.0×10^6 cells). Where indicated, 0.19 μ M (TA3) or 0.28 μ M (TA3-MTX-R) CCCP were added (Fones et al., 1989).

2.6. Growth inhibition of TA3 and TA3-MTX-R cell lines

Both cell lines were cultured in the absence and presence of amine C (*N*-(3-methoxy-4-hydroxybenzyl)-*p*-toluidine) in Dulbecco's modified Eagle's medium with 7% fetal bovine serum, 25 mM Hepes [4-2(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 44 mM NaHCO₃, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. For the experiments, $1.8\text{--}2.2\times 10^6$ cells/ml were seeded in 20 ml of culture medium, in 125 ml culture flasks, and grown at 37 °C for up to 96 h. The cells were first allowed to grow for 24 h (about 4.0×10^6 cells/ml) and amine C was then added. Cell numbers were determined every 24 h with a Neubauer counting chamber and cell viability was determined by trypan blue exclusion.

2.7. Preparation of mitochondria

Mitochondrial suspensions of about 40–50 mg protein/ml were prepared from tumour cells (Moreadith and Fiskum, 1984) and from mice liver (Ferreira and Gil, 1984), with the following minor modifications: mitochondrial fractions were washed twice at $12\,000\times g$ for 10 min and resuspended in a minimal volume of their respective medium in the absence of bovine serum albumin to eliminate adsorption of hydrophobic molecules. Protein concentration was determined by the Lowry reaction as described above.

2.8. Mitochondrial oxygen consumption

To determine the effects of the imine and amine derivatives of vanillin on oxidative phosphorylation, oxy-

gen consumption was monitored polarographically with a Clark electrode at 25 °C. The respiration medium contained: 200 mM sucrose, 50 mM KCl, 3 mM K₂PQ, 4 mM MgCl₂, 0.5 mM EGTA and 3 mM Hepes, pH 7.4; the substrates: 2.5 mM glutamate plus 2.5 mM malate or 5.0 mM succinate and 0.25 mM ADP. The mitochondrial suspensions (2 mg) were incubated for 2 min at 25 °C before adding each derivative. All measurements were made after 5-min pre-incubation with each compound.

3. Results

We report the effects of a homologous series of four vanillin derivatives on respiratory rates of TA3, TA3-MTX-R and mouse hepatocytes. Effects of amine C on culture growth of both tumour cells are also described.

Fig. 2 shows effects of imine A on respiratory rates of TA3 and TA3-MTX-R cell lines. Sigmoidal inhibitory curves, which were fitted in accordance with the Hill equation (Monod et al., 1963), were observed when imine A concentration was increased in the assay system both in the absence and presence of the uncoupler CCCP. Maximal inhibition (80–85%) of the respiratory rates of both tumour cell lines in the absence of CCCP was at about 2 mM. Also, maximal inhibition (89–94%) of CCCP-stimulated respiration by both tumour cell lines was reached at about 2 mM imine A.

Very similar sigmoidal inhibitory curves were observed when amine C concentration was increased in the assay system with these two tumour cell lines (Fig. 3). Maximal inhibition (86–93%) of respiratory rates of both tumour cell lines was at about 1 mM. The inhibition was slightly higher with uncoupler CCCP than in its absence, but differences were statistically insignificant. Only slight variations in the levels of inhibition were found for these two tumour cell lines. However, amine C was significantly more effective than imine A. These results also suggest that the level of inhibition, although dependent on the amine/imine concentration, is not predominantly dependent on respiration being coupled to ADP phosphorylation. Consequently, the primary interaction occurs in the electron transfer pathway through the mitochondrial respiratory chain.

Table 1 summarizes effects of four-vanillin chemical analogues on the rate of oxygen consumption of TA3 and TA3-MTX-R tumour cells. Concentrations of the compounds necessary to inhibit 50% of the respiratory rate (IC_{50}) were 0.22–3.87 mM. Generally, IC_{50} for vanillin was higher than that of its derivatives and amines were more active than imines toward both lines in the absence or presence of the uncoupler CCCP.

To establish the inhibitory site of these compounds within the mitochondrial electron transport chain of intact tumour cells, we selected the two derivatives with highest

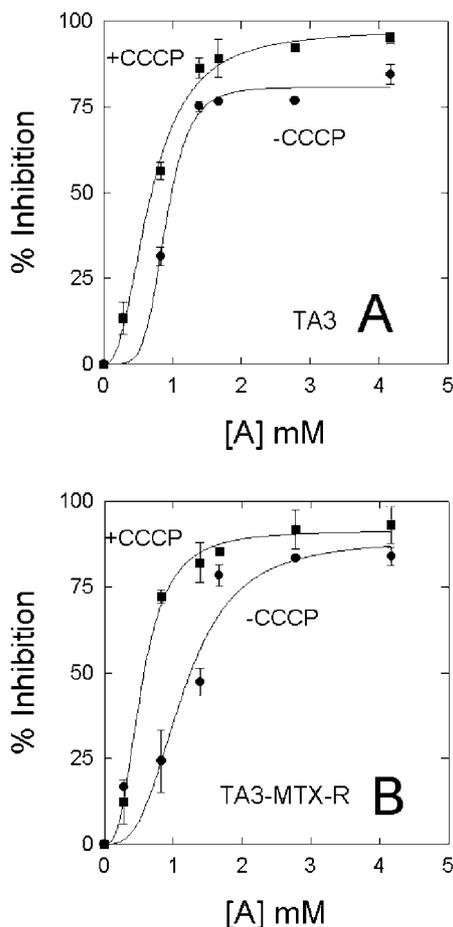


Fig. 2. Effect of imine A concentration on the respiratory rate of TA3 and TA3-MTX-R tumour cell lines in the absence and presence of CCCP. Results are expressed as percentage of inhibition of the oxygen consumption rate compared with the respective control in the absence of imine A. Control activities, expressed as nmol/min per mg protein, were: 9.46 ± 1.94 (-CCCP) and 25.8 ± 4.57 (+CCCP, $0.14 \mu\text{M}$) for TA3 cell line; 8.06 ± 1.28 (-CCCP) and 25.2 ± 2.70 (+CCCP, $0.28 \mu\text{M}$) for TA3-MTX-R cell line. Each result is the mean \pm S.D. of at least three independent determinations. Data were plotted in accordance with the Hill equation. Further details in Section 2.

inhibitory action. Their effects on the respiratory rate were determined in the presence of well-characterized synthetic substrates that donate electrons to the energy-conserving sites 2 (duroquinol) and 3 (TMPD). Fig. 4 shows the cellular respiration of TA3 tumour cells in the presence of different mitochondrial electron flow inhibitors and two vanillin derivatives. Rotenone, antimycin A, imine A and amine C inhibited oxygen consumption. However, cellular respiration was newly observed by the addition of ascorbate plus TMPD, indicating that inhibition does not involve Complex IV. Results were similar for the TA3-MTX-R cell line. Moreover, the ascorbate plus TMPD oxidation rate was higher in the presence of imine A than in the presence of rotenone, antimycin A or amine C, which would suggest that imine A has a slight uncoupling effect on the respiratory chain. To explore this action, we

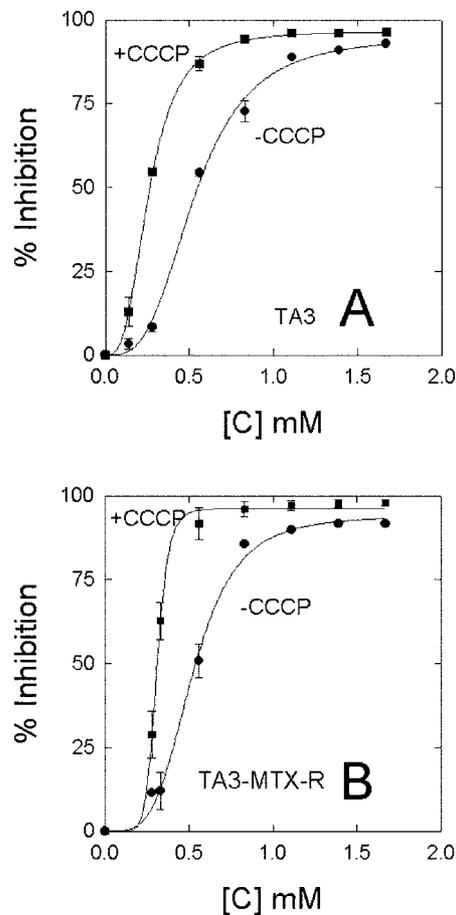


Fig. 3. Effect of amine C concentration on the respiratory rate of TA3 and TA3-MTX-R tumour cell lines both in the absence and presence of CCCP. Results are expressed as percentage inhibition of oxygen consumption rate compared with the respective control in the absence of amine C. Controls activities, expressed as nmol/min per mg protein, were: 9.14 ± 1.81 (-CCCP) and 25.2 ± 1.08 (+CCCP) for TA3 cell line; 9.22 ± 0.99 (-CCCP) and 26.6 ± 4.23 (+CCCP) for TA3-MTX-R cell line. Each result is the mean \pm S.D. of at least three independent determinations. Other conditions are described in Fig. 2. Further details in Section 2.

performed an experiment where imine A was added after addition of oligomycin, rotenone and ascorbate plus TMPD (Fig. 4, trace fourth). Imine A increased the

Table 1
Effects of vanillin and its derivatives on cell respiration rates in the absence and presence of the uncoupler CCCP

	IC ₅₀ ^a (mM) TA3		IC ₅₀ ^a (mM) TA3-MTX-R	
	-CCCP	+CCCP	-CCCP	+CCCP
Vanillin	2.51 ± 0.05	3.32 ± 0.08	3.87 ± 0.09	0.72 ± 0.02
A	1.07 ± 0.01	0.75 ± 0.01	1.42 ± 0.04	0.63 ± 0.01
B	2.25 ± 0.05	1.00 ± 0.04	2.16 ± 0.06	0.22 ± 0.01
C	0.53 ± 0.02	0.26 ± 0.01	0.56 ± 0.03	0.31 ± 0.02
D	0.74 ± 0.02	0.42 ± 0.01	0.69 ± 0.03	0.50 ± 0.03

^a IC₅₀ corresponds to the concentration of assayed compounds necessary to inhibit cell respiration by 50%. Values are arithmetical means \pm S.D. of at least three independent determinations.

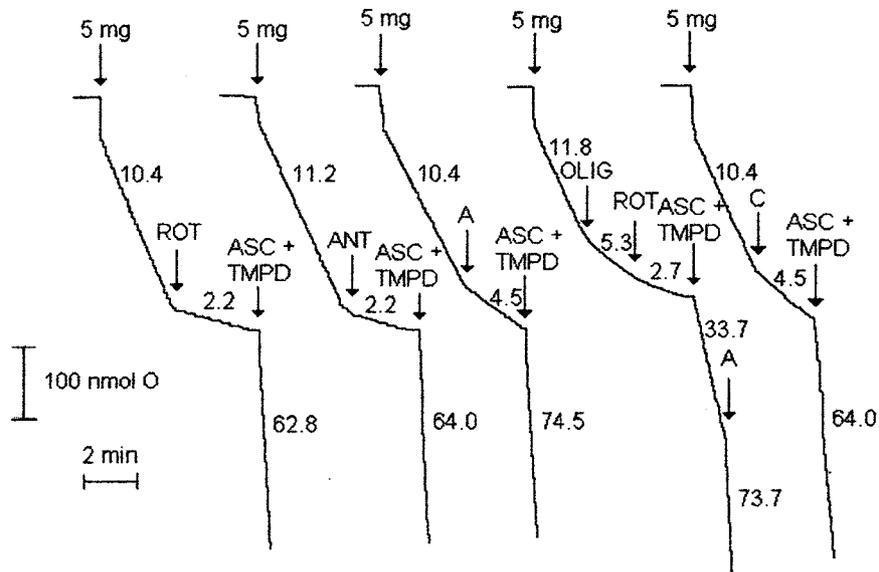


Fig. 4. Effect of TMPD on the respiratory rate of intact tumour cells of TA3 line previously blocked with some vanillin derivatives. Arrows indicate where TA3 cell line (5.0 mg of proteins and the final concentration of 5.6 mM glutamine as substrate), imine A (1.4 mM, A), amine C (0.8 mM, C), rotenone (2.2 μ M, ROT), antimycin (0.6 μ g/ml ANT), ascorbate (5.6 mM, ASC) plus TMPD (1.0 mM) and oligomycin (1.0 μ g/ml, OLIG) were added to the respiration medium. Numbers indicate oxygen consumption rates as nmol/min per mg protein. Further details in Section 2.

respiratory rate about 2-fold, indicating a slight uncoupling.

Fig. 5 shows that duroquinol oxidaton was inhibited by antimycin A, but not by rotenone, imine A nor amine C. These results not only support the conclusion that imine A and amine C inhibit electron flow at some point before ubiquinol:cytochrome c oxidoreductase, but they also indicate that these compounds do not inhibit electron flow from Complex III to oxygen. Similarly, these compounds,

like rotenone, interfere with components of electron transfer, presumably at some site of NADH dehydrogenase. Since the addition of imine A or amine C caused an immediate large increase in absorbance at 340–370 nm, it may be concluded that NAD(P)⁺ became more reduced when these compounds were added (results not shown). Moreover, the duroquinol oxidation rate is higher in the presence of imine A than with rotenone or amine C. Imine A increases oxygen consumption, which was previously

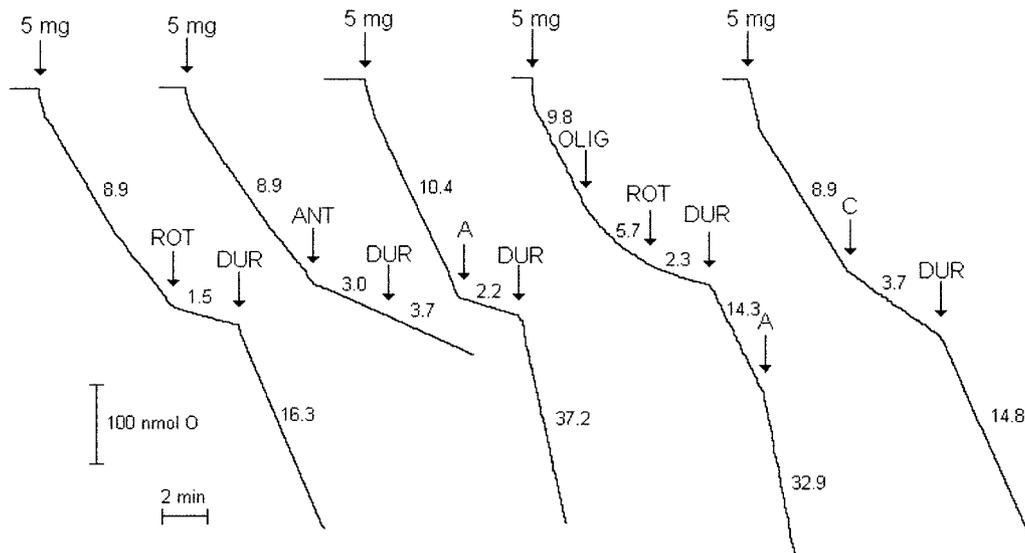


Fig. 5. Effects of imine A and amine C on duroquinol oxidation rate in TA3 tumour cell line. Arrows indicate where TA3 cell line (5.0 mg of proteins and the final concentration of 5.6 mM glutamine), imine A (1.4 mM), amine C (0.8 mM), rotenone (2.2 μ M), antimycin (0.6 μ g/ml), duroquinol (1.0 mM, DUR) and oligomycin (1.0 μ g/ml) were added to the respiration medium. Numbers indicate oxygen consumption rates as nmol/min per mg protein. Further details in Section 2.

inhibited by oligomycin (Fig. 5, trace fourth), suggesting that imine A has a slight uncoupling effect on the respiratory chain.

Fig. 6A shows the effect of amine C on the rate of succinate oxidation in permeabilized TA3 cells. Respiratory rate inhibition of about 75% of the control was obtained at 0.8 mM amine C, indicating that electron flow from succinate to ubiquinone (Complex II) was also inhibited in intact cells. Therefore, we also determined the effect of amine C concentration on the respiratory rate in the presence of succinate as a substrate of both tumour cell lines. Very similar sigmoidal inhibitory curves were observed when amine C concentration was increased in the assay system (Fig. 6B,C). Maximal inhibition (88–95%) of oxygen consumption rates of both TA3 and TA3-MTX-R cell lines was attained at about 1.5 mM. The inhibitory effect was slightly higher with CCCP, but differences were statistically insignificant. There were only slight variations in levels of inhibition in these two tumour cell lines.

Table 2 summarizes the effects of amine C on oxygen consumption rates of both tumour cells and mouse hepatocytes and also of their respective mitochondrial fractions. When NAD⁺-linked substrates were oxidized, IC₅₀ values of oxygen consumption rates by hepatocytes (5 mg of protein) and mouse liver mitochondria (2 mg of protein) were very similar, in agreement with the assumption that about 40% of the total cellular mass corresponds to mitochondrial mass. Instead, IC₅₀ values for both intact tumour cells were approximately 2.5 times higher than those calculated for respective mitochondria fractions from TA3 and TA3-MTX-R tumour cells. These differences were higher than expected. Plausible explanations could be that the intramitochondrial amine C concentration in intact tumour cells is lower than in the surrounding mitochondrial respiration medium, that amine C does not easily enter the tumour cell at pH 7.4, or that it is tightly bound to membranes other than the mitochondrial inner membrane. In addition, both TA3 and TA3-MTX-R tumour cells were about 4-fold more sensitive to amine C than mouse hepatocytes. This difference should increase if results are expressed per cell number, since we counted about 1.15×10^6 mouse hepatocytes per mg of protein and about 1.95×10^6 tumour cells per mg, which might explain the fact that IC₅₀ values of respiratory rates by mitochondria isolated from both tumour cells were about 10-fold lower than those from mouse liver. Amine C also inhibited succinate oxidation by tumour cells and by hepatocytes (non-malignant cells), the former being about 4-fold more sensitive than hepatocytes; but amine C did not inhibit oxygen consumption rates in all mitochondrial fractions. Probably, amine C can be biotransformed in the cell to a compound which blocks electron flow through the succinate dehydrogenase-CoQ span. The effect of amine C on non-malignant cells suggests that the other derivatives could also be, by extension, of pharmacological value.

Fig. 7 shows time-course effects of amine C on the

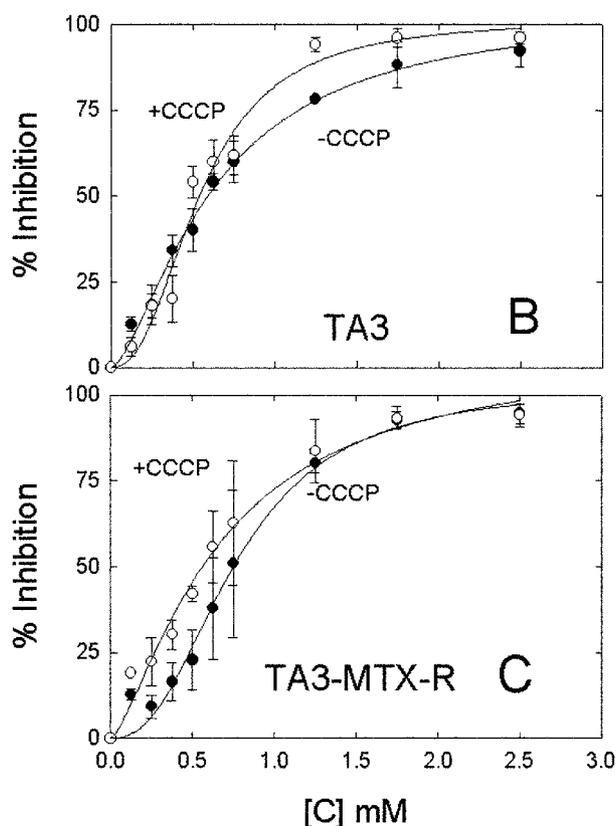
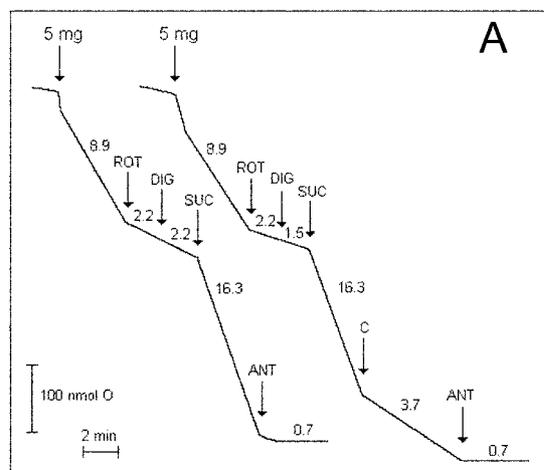


Fig. 6. Effects of amine C on succinate oxidation rates in TA3 and TA3-MTX-R tumour cell lines. In (A), arrows indicate where TA3 cell line (5.0 mg of proteins and 5.6 mM glutamine), rotenone (2.2 μ M), digitonin (0.006%), succinate (5 mM, SUCC), amine C (0.8 mM) and antimycin (0.6 μ g/ml) were added to the respiration medium. Numbers indicate oxygen consumption rate as nmol/min per mg protein. Control activities, expressed as nmol/min per mg protein, were: 16.5 ± 1.11 (-CCCP) and 19.80 ± 2.03 (+CCCP) for TA3 cell line (B); 13.40 ± 1.40 (-CCCP) and 21.70 ± 3.20 (+CCCP) for TA3-MTX-R cell line (C). Each result is the mean \pm S.D., $n=1$ of at least three independent determinations. Data were plotted in accordance with the Hill equation. For other details, see Section 2.

growth of both tumour cells, which are concentration-dependent for both lines. At the lowest concentration (5 μ M) and after 24 h of exposure, cell viability was 79% for

Table 2

Effects of amine C on respiratory rates of tumour cells, mouse hepatocytes, and mitochondria isolated from mice liver, TA3 and TA3-MTX-R cell lines

	Mitochondria		Cells	
	Glutamate + Malate IC μM	Succinate % inhibition	NAD ⁺ -linked Substrates ^a IC μM	Succinate IC μM
TA3	0.23±0.01	<50	0.53±0.03	0.59±0.05
TA3-MTX-R	0.20±0.03	<50	0.56±0.04	0.75±0.16
Mice Liver	2.05±0.10	<50	2.07±0.35	2.48±0.73

^aGlutamine (5.6 mM) (tumour cells) or 10 mM glucose plus 5.6 mM glutamate (mouse hepatocytes).

IC₅₀ corresponds to the concentration of the assayed compounds necessary to inhibit cell respiration by 50%. Values are arithmetical means±S.D. of at least three independent determinations.

TA3 and 70% for TA3-MTX-R cells. However, cell viability decreases significantly after 72 h with the highest concentration (80 μM), i.e., by an average of 11 and 17% for TA3 and TA3-MTX-R cell lines, respectively.

Results on culture growth of both tumour cell lines gave an IC₅₀ of 18.6 μM after 24 h for amine C, and of 11.4 μM after 48 h for TA3 cells. For the TA3-MTX-R cells,

IC₅₀ of amine C was 14.1 μM at 24 h and 13.4 μM at 48 h.

4. Discussion

Results show that amine C is the best respiratory inhibitor among the four derivatives of vanillin. These derivatives primarily inhibit NADH re-oxidation catalyzed by the mitochondrial respiratory chain of TA3, TA3-MTX-R tumour cells and mouse hepatocytes, since oxygen consumption is similarly inhibited both in the absence and presence of CCCP (Morello et al., 1995). In the absence of CCCP, inhibition curves are sigmoidal, showing an inhibitory cooperative effect on electron flow, which would suggest the existence of more than one inhibition site (Fones et al., 1989; Pavani et al., 1994). In the presence of CCCP, the sigmoidal nature of the inhibition curves decreased and a stronger inhibitory effect occurred. Plausible explanations could be that: (1) respiration in the coupled state of mitochondria is limited by endogenous controllers. Although there is never a single 'rate limiting step', the system qualitatively behaves so that marked inhibition is only observed when exogenous inhibition exceeds the control exerted by the endogenous controller, in this case proton permeability of the inner mitochondrial membrane. The result is a pseudo-sigmoidal inhibition curve. Thus, the observed difference is understandable. (2) The inhibitory activities of these compounds depend on some particular redox condition of the mitochondrial respiratory chain components. The uncoupler induces the respiratory chain components to reach a more oxidized stationary state. Thus, these vanillin derivatives are better inhibitors when electron carriers change towards a relatively more oxidized state (Morello et al., 1995). (3) If a compound behaves as an uncoupler as well as an inhibitor, both inhibition and stimulation of respiration can occur simultaneously in the 'coupled' cells due to the uncoupling effect, leading to an apparent lower sensitivity (Floridi et al., 1994).

Imines and amines lessen electron flow mainly through the NADH-CoQ span, since when succinate is the substrate of isolated mitochondria there is no major inhibition

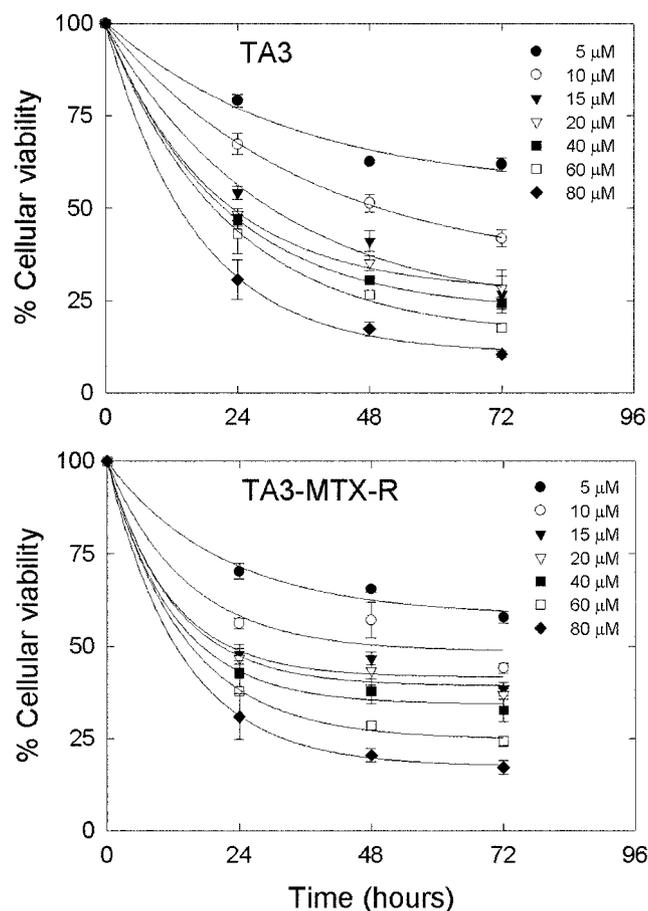


Fig. 7. Effects of amine C on TA3 and TA3-MTX-R tumour cell culture growths. Results are presented as percentage of cellular viability compared with the respective control and measured every 24 h. They are the arithmetical means±S.D. (error bars) of triplicate determinations of at least three independent experiments. Amine C was added at seven different concentrations. Further details in Section 2.

(less than 50%); however, amine C inhibits succinate oxidation in permeabilized intact tumour cells, indicating possible alteration of electron flow through complex II (Fig. 6). This brings up the possibility that these compounds are metabolized to a catechol-like compound. While *O*-demethylation is catalyzed by the cytochrome-P-450 family of enzymes placed in the smooth endoplasmic reticulum, such reactions will likely occur in TA3 and TA3-MTX-R cell lines. These compounds may be demethylated to some extent by intact cells, but not by mitochondrial fractions, thus also inhibiting succinate oxidation. Nordihydroguaiaretic acid, a di-catechol, is an electron flow inhibitor through the both complex I and complex II of the respiratory chain (Pavani et al., 1994; Shi and Pardini, 1995).

Amines are better inhibitors than imines. Imines have conjugated double bonds and they are more rigid than amines, which can order rings spatially and interact better with targets of the respiratory chain (Satoh et al., 1996). Amine C is a slightly better respiratory rate inhibitor than amine D. As it has an extra methyl group bound to the benzene ring it is more hydrophobic, causing a better interaction with the mitochondrial inner membrane.

Variations in IC₅₀ in the intact tumour cells may be due to possible differences in rigidity of the mitochondrial membrane in the tumour lines under study, because contents of mitochondrial membranes are variable and specific to each kind of tumour cells (Pedersen, 1978). Also, large membrane variations have been observed when multiresistance occurs (Pickard and Kinsella, 1996).

In the first 24 h exposure, amine C at low concentration inhibits growth, the TA3 cell line being less sensitive than TA3-MTX-R. However, at higher concentrations, there are no important differences between the cell lines, probably because of its better absorption rate and intracellular distribution. After 24 h, the TA3-MTX-R line was more sensitive than the original one; however, this effect changed after 48 h, probably due to differences in mechanisms as absorption rate, intracellular distribution, metabolism and excretion of amines. These variations can also be due to changes in rigidity of mitochondrial membranes of tumour lines characteristic of each cell line (Pedersen, 1978). Results from cell growth and oxygen consumption appear consistent, because the assays to determine amine effects on respiration were performed with nearly 10⁶ cells/ml. Instead, cell growth determinations were carried out with about 0.4⁶ cells/ml. Therefore the amine amount is equivalent to the number of cells.

These compounds would inhibit oxidative phosphorylation, preventing ATP synthesis, thus stopping cellular processes that require energy, and starting a complex chain of events resulting in cellular death. These findings would partly explain the antineoplastic activity of the synthesized derivatives.

The main obstacle to the successful treatment of neoplastic diseases is that tumours often exhibit intrinsic or

inherent resistance to chemotherapeutic agents, or that they may develop resistance to treatment after the process of an initial response (acquired resistance), subsequently becoming insensitive to a range of anti-cancer agents. The TA3-MTX-R cell line is resistant to various agents, such as doxorubicin and vinblastine (substrates of P-glycoprotein); as well as, methotrexate, cisplatin and 5-fluorouracil (non-substrates of this multidrug transporter). IC₅₀ values for tumour mitochondria from both cell lines and mice liver point to a selectivity of amine C at the mitochondrial level. Amines behave similarly for both cell lines, which implies similar sensitivity for these tumour cells. Thus, these derivatives might be further considered as potential anti-neoplastic drugs.

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