Targets of 3-Bromopyruvate, A New, Energy Depleting, Anticancer Agent

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Abstract: 3-bromopyruvate (3-BrPA), a pyruvate analog recently proposed as a possible anticancer drug, was investigated in relation to its capacity to inhibit energy production in fractions obtained from normal cells (rat hepatocytes) and in isolated rat thymocytes. Findings were that main targets of the drug were glyceraldehyde 3-phosphate dehydrogenase, and not hexokinase as suggested for hepatoma cells, and succinate -driven ATP synthesis. Consistently with the above findings, in the normal cells studied (thymocytes) the drug elicited an important fall in ATP levels. The significance of the present findings in concern with a possible therapeutic usefulness of the drug is discussed.

Keywords: 3-Bromopyruvate, Warburg effect, tumor therapy, glycolysis, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, oxidative phosphorylation, succinate dehydrogenase.

INTRODUCTION

3-BrPA has recently been reported to eradicate liver cancer in animals without associated systemic toxicity [1-3]. This notable effect has been attributed to selective inhibition of hexokinase, although oxidative phosphorylation has also been reported to be inhibited [1]. As yet, possible other targets of the drug, as an energy blocker, in addition to hexokinase, have not been investigated. Thus, the molecular mechanism involved in drug action remains incompletely characterized and, more relevant, still needs to be determined why the drug is not toxic for normal cells. Therefore, also considering that 3-BrPA is a strong alkylating agent [4], it was thought to be of interest to examine the effect of 3-BrPA in non- transformed cells on hexokinase and other glycolytic enzymes that could also be involved, as well as on mitochondrial ATP production. In the present study, the cytosolic and mitochondrial fractions, obtained from rat liver and isolated rat thymocytes were used to address the above questions. Our findings indicate that in normal cells, the main target of 3-BrPA in the glycolytic pathway is not hexokinase, as it is thought to be for liver cancer cells (1), but enzyme glyceraldehyde 3–phosphate dehydrogenase, as reported in other studies [5, 6]. Moreover, succinate driven ATP synthesis in isolated rat liver mitochondria was inhibited by 3-BrPA. As expected, ATP levels in isolated cells were significantly reduced by 3-BrPA.

MATERIALS AND METHODS

Preparation of a Cytosolic Fraction from Rat Liver

A rat liver homogenate obtained in a sucrose medium was submitted to standard centrifugations to eliminate debris, nuclei, unbroken cells and mitochondria. The resulting supernatant was submitted to centrifugation for 30 minutes at 30000 g to eliminate residual mitochondria, lysosomes and other particulate material. All procedures were conducted at about 4 ^{o}C. The supernatant was diluted many fold, as specified in the legend to the figures, in a medium containing KCl 130 mM, $MgSO_4$ 5 mM, Hepes 20 mM pH 7.4, or in a medium containing K^+pirophosphate 0.1M pH 8.5 as specified in the legends. The protein content of the final cytosolic suspension was determined by the Bio-Rad protein assay using bovine serum albumin as standard.

Cell and Organelle Preparations

Rat thymocytes were prepared by a standard procedure and suspended in a simplified saline containing 135 mM NaCl, 5 mM KCl, 5mM $MgSO_4$, 5mM $NaHCO_3$ 30 mM HEPES buffer pH 7.3 and 5 mM glucose or not glucose as indicated and maintained at room temperature (22 ± 2 °C).

Rat liver mitochondria were isolated by differential centrifugation in a medium containing 250 mM sucrose, 5 mM Tris-HCl, 250 µM EDTA pH 7.8 [7]. Liver submitochondrial particles (SMP) were prepared by means of a procedure that involves sonication of rat liver mitochondria in a hypotonic medium as described (reference in [8]) and suspended at the last step of the preparation in 0.25 M sucrose, 20 mM Hepes pH 7.4. A different medium, see below, was used for acidification assays. Protein was determined as above.

Assays

Lactate dehydrogenase was assayed in the cytosolic suspension described above supplemented with NADH. The reaction was initiated by adding pyruvate or 3-BrPa as indicated. NADH consumption was followed fuorimetrically: the reduced form of NAD^+ absorbs light at 340 mµ and emits a fluorescence band at a longer wavelength which has a peak at about 465 mµ. The above wavelengths were selected for excitation and emission respectively (± 10 mµ as pass-band), using a Perkin Elmer MPF 2A spectrofluorimeter. See other details in the legend to Fig. (**1**).

Hexokinase was assayed in the cytosolic fraction diluted with the KCl medium described above, in the presence of 1mM KCN, 6 µM rotenone. Reaction was started by adding consecutively $NADP^+$, glucose and ATP. The dinucleotide reduction was followed fluorimetrically; employing excita-

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tion and emission wavelengths as described for lactate dehydrogenase assay. This assay couples the glucose-6-phosphate formed in the hexokinase reaction to the glucose-6-phosphate dehydrogenase [9]. See details in the legend describing the assay.

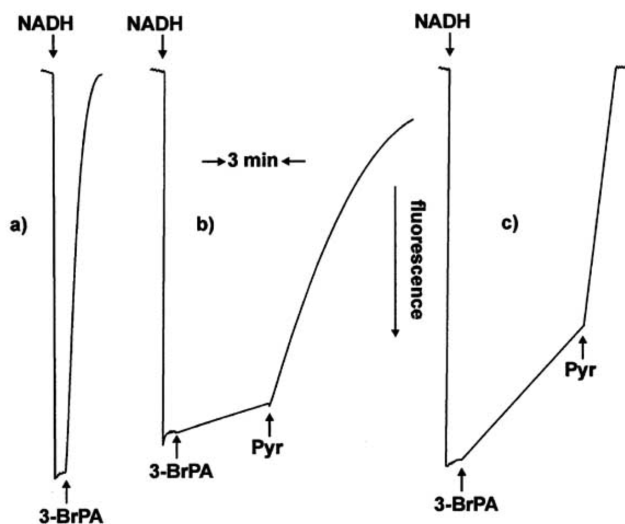

Fig. (1). 3-BrPA is a substrate for lactate dehydrogenase. Comparison with pyruvate.
NADH oxidation was monitored fluorimetrically as described in Materials and Methods. The supernatant obtained from a rat liver homogenate was diluted with a KCl medium to a final protein content of about 1.5 mg/ml (total volume 2 ml) as described in Materials and Methods. Additions (final concentrations): **a)** NADH 250 μM, and successively 3-BrPA 500 μM **b)** the above suspension contained: oxamate 35 mM; NADH and 3-BrPA additions as in **a)**; then 500 μM pyruvate (pyr). **c)** the suspension contained oxamate 11 mM; other addition as in **b)**.

Glyceraldehyde 3-phosphate dehydrogenase was assayed in the cytosolic suspension described above, diluted in a 0.1 M K^+-pirophoshate, pH 8.5 medium, supplemented with NAD^+ [10].

The reaction was initiated with glyceraldehyde 3-phosphate and NAD^+reduction was followed fluorimetrically. Other additions and details are given in the legend of the figure describing the assay. NAD^+, as soon as it was added (Fig. **2**), was slowly reduced by a reaction not ascribable to glycolytic enzymes as it was not inhibited by oxamate or iodoacetamide; moreover, following the glyceraldehyde induced NAD^+ reduction, a NADH oxidation was observed. The first effect was tentatively ascribed to fatty acid oxidation or contamination from broken mitochondria, the latter, also observed in the presence of iodoacetamide, was ascribed to glycerol 3-phosphate dehydrogenase activity following conversion of glyceraldehyde 3-phosphate in dihydroxyacetone phosphate. Nevertheless, both of the above effects did not compromise the determination of glyceraldehyde 3-phosphate dehydrogenase activity that was marked by iodoacetamide inhibition.

Mitochondrial ATP synthesis assay was performed in the KCL medium described above, supplemented with 100 μM diadenosine 5' pentaphosphate (AP_5A), 10 mM K_2HPO_4, 5.5 mM glucose, 6 μM rotenone, 500 μM $NADP^+$ and the coupled enzymes hexokinase and glucose-6-phoshate dehydrogenase (both 2.5 -3 unit /ml) [9]. Reaction was started with 1.5 mM ADP and 10 mM succinate (final concentrations) added consecutively and the $NADP^+$ reduction was followed fluorimetrically.

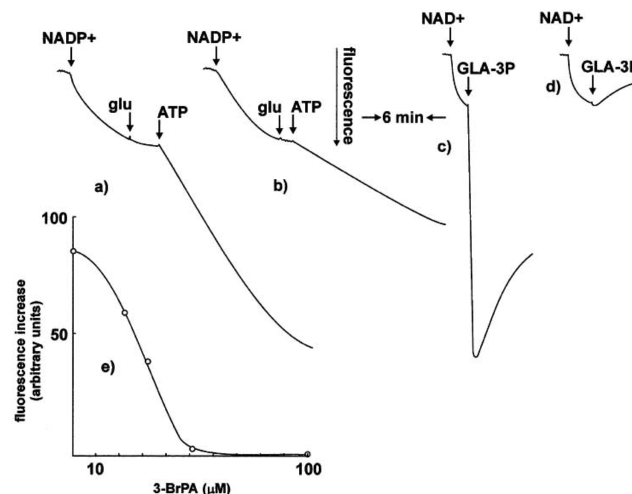

Fig. (2). Inhibition of hexokinase and glyceraldehyde 3-phosphate dehydrogenase by 3-BrPA.
a), b) hexokinase activity was determined as described in Material and Methods. As shown in trace **a)** and **b)** $NADP^+$ addition (500 μ M, final concentration) to the suspension described in fig 1, resulted in a partial reduction of the dinucleotide due,presumably, to metabolites present in the suspension: ; glucose, followed by ATP addition, elicited an extensive further $NADP^+$ reduction. Protein in the diluted cytosol (KCl medium) 2.2 mg/ml about; in b) 3- BrPA 500 μM was present 3-BrPA had no effect on the activity of glucose 6-phosphate dehydrogenase as verified by adding glucose 6-phosphate in the place of ATP plus glucose (not shown). **c) d) e)** Glyceraldehyde 3-phosphate dehydrogenase was determined as described in Materials and Methods. Tentative explanation for the slight initial reduction of NAD^+ and the partial reversal observed at the end of the glyceraldehyde induced NAD^+ reduction phase (**c** and d), is described in Materials and Methods. The medium (k^+pirophosphate 0.1 M, pH 8.5) contained in addition: 10 mM sodium arsenate, 625 μM NAD^+, 1mM KCN, 6 μM rotenone and about 0.9 mg/ml of cytosolic protein. The reaction was started with 150 μM glyceraldehyde 3-phosphate (GLA 3-P; final concentration). **d)** 0.5 mM iodoacetamide or 0.5 mM 3-BrPa was present in the assay **e)** determination of the Ki for the inhibition of the enzyme by 3-BrPA evaluated by determining the initial rate of NAD^+ reduction.

Mitochondrial ATPase was monitored as proton pump activity in SMP by following the ATP- driven setting up of a pH gradient in SMP (acidic interior) [8] using the basic dye acridine orange (AO) as a probe. Accumulation of the dye in response to the pH gradient was detected fluorimetrically [11] ; the dye was excited at 492 mμ and fluorescence emission at 530 mμ (pass-band ± 10 mμ) was collected using a Perkin Elmer MPF 2A spectrofluorimeter.

Cell ATP content was estimated by monitoring fluorimetrically the reduction of (externally added) $NADP^+$ in the presence of glucose, and the coupled enzymes, hexokinase and glucose -6-phosphate dehydrogenase as described above

for the ATP synthesis assay. The reaction was started by adding Triton X 100 (0.05 %, final concentration) to liberate ATP from the cells. Iodoacetamide 1 mM, potassium cyanide 1 mM and 100μM AP_5A were also added just before Triton to block ATP synthesis. As a control, ATP was at times added at the end of the $NADP^+$ reduction phase, initiated by Triton X 100 addition, to calibrate the fluorimetric signal or validate the assay for the different medium manipulations used.

Control experiments (see also Results) indicated that 3-BrPA at the maximal concentration used (0.5 mM) had no effect on the activity of the enzymes (hexokinase and glucose 6-phosphate dehydrogenase (from Saccharomyces cerevisiae), used in the assay.

Each assay was performed at room temperature; data are representative of at least 3 independent experiments. Protein determination was made as above.

Chemicals

3-BrPA was prepared as a 0.1 M H_2O solution; this was acidic (pH 2.5 about). However, 3-BrPA additions, up to 1 mM, modified the cell or organelle suspension pH by not more than 0.1 unit. All chemicals were from SIGMA.

RESULTS

3 -BrPA is a Substrate for Lactate Dehydrogenase

3-BrPA elicited, in the cytosolic fraction of rat liver described in Materials and Methods, NADH oxidation (Fig. **1a**) at a much lower rate than that recorded using the physiological substrate pyruvate, which caused nearly immediate NADH oxidation (not shown). NADH oxidation could be ascribed to lactate dehydrogenase activity as it was nearly completely prevented by oxamate, a known inhibitor of the enzyme, in the case of 3-BrPA, while the rate was strongly reduced with pyruvate (Fig. **1b**). Using a concentration of oxamate producing only a partial inhibition (11 mM) it was possible to obtain an approximative estimation of the relative affinity of the two substrates for the enzyme lactate dehydrogenase ; the result was (Fig. **1c**) that 3-BrPA affinity was 9-11 times less than that of pyruvate (as determined in 9 trials using two distinct preparations).

Thus, 3-BrPA can compete with pyruvate for lactate dehydrogenase activity, that presumably transforms it into 3-Brlactate. The role of this reaction in glycolysis inhibition by 3-BrPa is discussed below.

3-BrPA Effect on Hexokinase

The cytosolic fraction from rat liver possesses hexokinase and glucose 6-phopshate dehydrogenase activities for, after a slight initial reduction of $NADP^+$, added to the suspension, a subsequent addition of ATP and glucose elicited an important further $NADP^+$ reduction (Fig. **2a**).

3-BrPA partially inhibited the glucose-ATP stimulated $NADP^+$ reduction, (Fig. **2b**); the Ki resulted nearly 400-500 μ M (determination not shown). This inhibition involved hexokinase and not glucose 6-phosphate dehydrogenase as it was not observed when the reaction was initiated with glucose 6-phosphate (not shown).

Inhibition by 3-BrPA of Glyceraldehyde 3-Phosphate Dehydrogenase

The reaction was followed as NAD^+ reduction, following the addition of glyceraldehyde 3 –phosphate in the presence of arsenate (Fig. **2**). As pointed out in Materials and Methods, the slight NAD^+reduction, before glyceraldehyde 3-phosphate addition, was presumably due to mitochondrial contamination (enzymes and substrates) and the reversal, observed following the reduction phase induced by glyceraldehydes 3-phosphat, tentatively ascribed to the cytosolic enzyme glycerol -3 phosphate dehydrogenase. Oxamate was added to the medium at a concentration (30 mM) able to prevent NADH oxidation by 3-BrPA via lactate dehydrogenase (see Fig. **1**). As expected, the reaction was inhibited by iodoacetamide (0.5 mM) well known inhibitor of this glycolytic enzyme. 3-BrPA at the same concentration equally completely inhibited NAD^+ reduction by glyceraldehyde 3-phosphate dehydrogenase (Fig. **2d**). Determination of the Ki for this inhibition gave a value of about 25 μM (Fig. **2e**). This inhibition was expected for 3-BrPA is a strong alkylating agent and thus mimics the action of iodoacetate.

3- BrPA Does Not Inhibit Mitochondrial ATPase

The possible effect of 3-BrPA on mitochondrial ATPase was studied by monitoring ATPase activity, acting as a proton pump in submitochondrial particles. In these artificial organelles which present an inside out conformation in respect to mitochondria, ATP drives H^+ -inward proton transport [8]. The resulting acidification of the lumen of the organelle was followed using the pH gradient probe acridine orange.

Unlike what happens to lysosomes [12], as expected oligomycin completely prevented the ATP elicited acidification (Fig. **3b**), while bafilomycin, specific inhibitor of H^+ vacuolar ATPase, except for a slight reduction in the rate, had no effect thus indicating that contamination from vacuolar ATPase was absent (Fig. **3c**). Unlike lysosome H^+ ATPase (vacuolar ATPase) [13)], mitochondrial ATPase was not inhibited by 3-BrPA (Fig. **3d**). This makes it likely that, also when operating in the inverse direction (ATP synthetase) the mitochondrial enzyme is not affected by 3- BrPA.

3-BrPA and Oxidative Phosphorylation

ATP synthesis was examined in isolated rat liver mitochondria fed with succinate in the medium described in Materials and Methods by following $NADP^+$ reduction to detect mitochondrial ATP synthesis, in the presence of the appropriate enzyme mixture. The assay was started by consecutively adding ADP and succinate. As shown, ADP elicited a slight fluorescence signal ($NADP^+$ reduction) as compared to that elicited by succinate. When both oligomycin and KCN were present in the medium, they nearly completely prevented ATP synthesis (Fig. **4b**). A similar inhibitory effect was elicited by 0.5 mM 3-BrPA (Fig. **4c**); the Ki for the inhibition by 3-BrPA was about 50 μM (Fig. **4e**) Since mitochondrial ATP ase activity was not inhibited by 3-BrPA (although the possibility exists that the enzyme remains inhibited when operating as ATP synthetase) the above result indicated that the Krebs cycle (or a segment of it, or downstream reactions) is another target of 3-BrPA Thus, at least, a

truncated Krebs cycle should be operative in the presence of 3-BrPA.

Instead, adenylate kinase, monitored in the above conditions(but in the absence of the inhibitor for this enzyme Ap_5A) and in the presence of oligomycin (Fig. **4d**), was not affected by 3-BrPA.

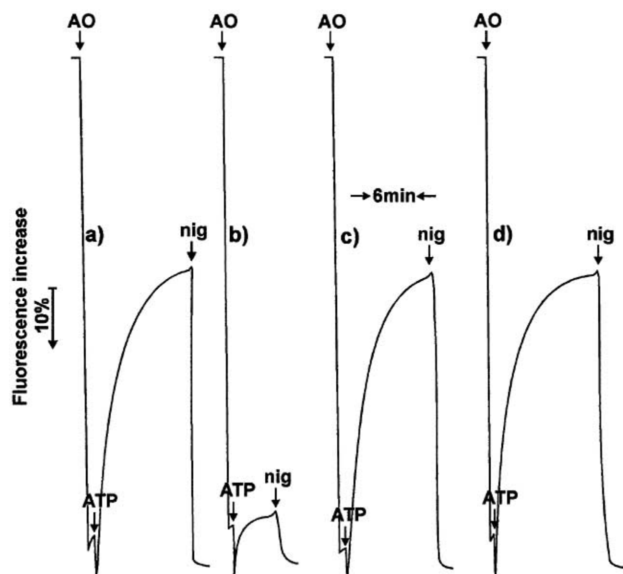

Fig. (3). 3-BrPA does not affect rat liver mitochondrial ATPase. H^+-transport, reflecting the activity of the mitochondrial ATPase, resulted in acidification, monitored as AO fluorescence decrease, of lumen SMP (0.25 mg/ml of protein; Bio-Rad assay) Medium: 125 mM KCl, 5mM $MgSO_4$, 20 mM HEPES pH 7.3. **a)** ATP-driven inward H^+ transport in SMP, monitored as AO fluorescence decrease, is shown. **b),c) d)** Inhibition by oligomycin (trace **b**) and insensitivity to bafilomycin and 3-brPA respectively (trace **c** and **d**). The medium contained a) no addition, b) 1.5 µg/ml oligomycin, **c) d)** 200 nM bafilomycin and 0.5 mM 3-BrPA respectively. Additions indicated in the figure: AO 5 µM, ATP 3 mM, nigericin 1 µg/ml (final concentrations).

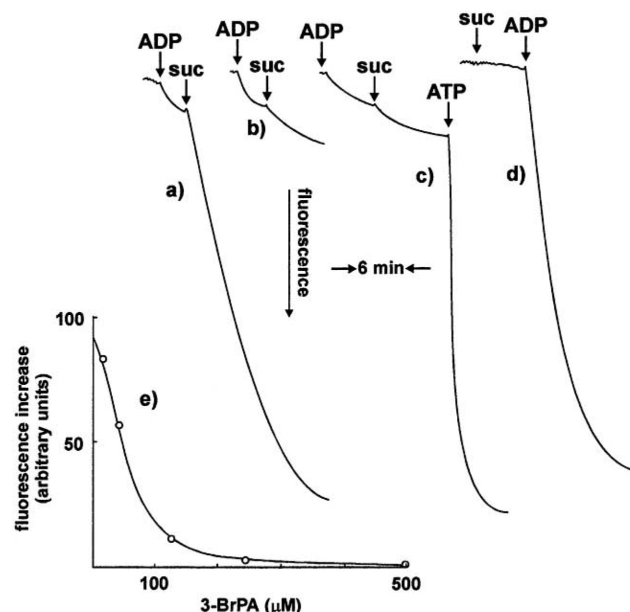

Fig. (4). Succinate- driven ATP synthesis inhibition by 3-BrPA. ATP synthesis was measured as described in Materials and Methods, by following fluorimetrically $NADP^+$ reduction. The reaction was started by adding consecutively ADP and succinate. Mitochondria protein in the assay mixture was about 0.5 mg/ml. **a)** no addition **b)** 3 µg/ml oligomycin and 1 m KCN were present **c)** 125 µM 3-BrPa was present; ATP was added to exclude interference of 3-BrPA with the ATP assay. **d)** the mixture contained 3 µg/ml oligomycin, 1 m KCN, 0.5 mM 3- BrPa, but not AP_5A **e)** : determination of Ki for the 3-BrPA inhibition of succinate driven ATP synthesis; rate of succinate -driven fluorescence increase (arbitrary unit) is plotted against 3-BrPA concentration. Additions (final concentrations): ADP 1.5 mM, Na succcinate (suc) 10 mM ATP, 580 µM.

DISCUSSION

Over 70 years ago, Warburg stated that glycolysis is enhanced in cancer cells also in the presence of oxygen (effect Pasteur absent) [14] Warburg's statement on the energy metabolism of cancer cells was in the successive decades submitted to numerous verifications that did not lead to its complete acceptance (for review see [15,16]). Nevertheless, as also pointed out by authors that have expressed some substantial criticism about the predominant or exclusive role of glycolysis in cancer cells to generate energy [16], the successful use of the positron emission tomography in clinical diagnosis of cancer clearly indicates that the glucose metabolism is profoundly altered in malignant cells. Therefore, the idea of using this cancer hallmark to contrast cancer cell growth has never been completely abandoned as demonstrated by the numerous reviews which have recently appeared on this topic [17-22]. Moreover, the cancer energy metabolism has recently earned a renewal of interest since it has been proven that much of the above cancer metabolic conversion is controlled by specific transcriptional programs [21].

Among the drugs able to impair cancer glucose metabolism, a prominent place has recently been given to 3-BrPA [1-3] a compound already known to impair glucose metabolism in certain normal cells [5, 6].

In studies demonstrating the usefulness of the drug against liver cancer, it has been suggested, that 3-BrPA is an inhibitor of glycolysis which works by inhibiting hexokinase II, an isoform of the enzyme present in tumour cells and prevalently bound to mitochondria [23].

The present study indicates that in normal cells (rat hepatocytes), hexokinase is not the preferred target of the drug which is glyceraldehyde 3- phosphate dehydrogenase, as elsewhere reported [5,6]. This result was expected for the drug is a strong alkylating agent [4]. Thus, 3-BrPA may act on glyceraldehyde 3-phosphate dehydrogenase as iodoacetate, the classic inhibitor of this enzyme, does.

However, in tumor cells that express a different isoform of the enzyme hexokinase [23], the above mentioned situation may not be true and this would explain the different sensitivity to the drug of normal cells as opposed to tumour cells [3].The different sensitivity among hexokinase isoenzymes to sulfhydryl inhibitors is well documented [24,25]. Nevertheless, the inhibition by 3-BrPA of glyceraldehyde 3-

phosphate dehydrogenase found in the present study, leaves the above question open once again.

On the other hand, the competition of 3-BrPA with pyruvate for lactate dehydrogenase cannot play a role in 3-BrPA toxicity for i) 3-BrPA affinity is nearly 10 times lower ii) in this reaction NAD^+ is formed allowing glycolysis to go on.

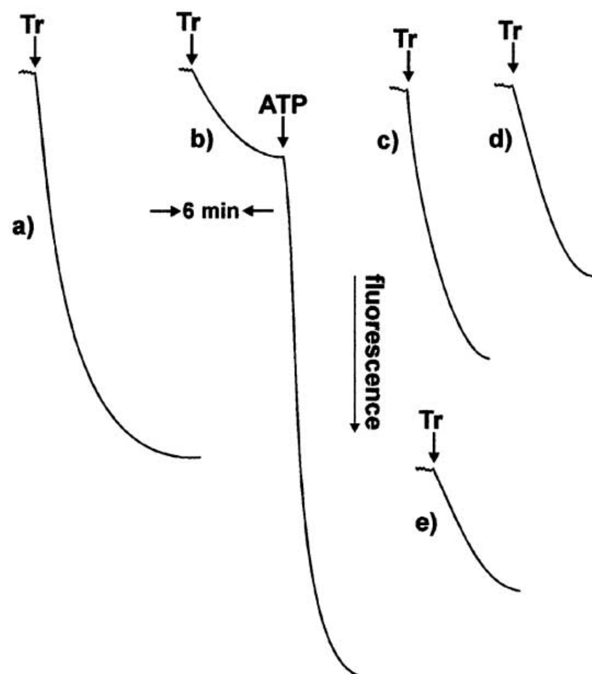

Fig. (5). ATP depletion elicited by 3-BrPA or metabolic inhibitors in rat thymocytes.
The cell suspension was maintained at room temperature in the simplified saline described in Materials and Methods supplemented with 5.5 mM glucose, and was divided in more aliquots which were submitted to different manipulations: trace **a)** no addition. Instead the medium contained: trace **b)** 0.2 mM 3-BrPA; trace **c), d)** 10 mM deoxyglucose, trace e) 10 mM deoxyglucose plus 3 µg/ml oligomycin (final concentrations). To initiate each assay, 2 ml aliquots were withdrawn from the above described cell suspensions for ATP determination, performed as described in Materials and Methods. The assays (trace **b,c,e**) were performed about 1 hour ; **d)** 3 hours following each of the above additions. Within the duration of the entire experiment (about 3 hours), viability of the cells exceeded 90%. Thymocytes, about 30-35 million /ml. Additions (final concentration) Triton X 100 (Tr) 0.05 %, ATP 60 µM. In b) ATP was added to exclude invalidation of the assay by 3-BrPA; the same fluorescence change was elicited by 60µM ATP in the absence of 3-BrPA and Triton (not shown).

The above described effects of the drug were of course expected to result in ATP content fall in intact cells. This has been observed in rat hepatoma cells [3, but also in rat thymocytes [13]. This finding was reconfirmed in the present study (Fig. **5**). One hour after addition, in rat thymocytes 3-BrPA elicited an important ATP level failure (up to 20-25 % of the initial value (Fig. **5b**)), greater than that elicited by deoxyglucose in the same time (only 20 % reduction by the glycolysis inhibitor, but a greater effect by deoxyglucose was observed after a few hours (Fig. **5c,d**)), while it was comparable to that elicited by oligomycin in combination with deoxyglucose (Fig. **5e**). This result was consistent with an inhibition by the drug involving both energy yielding pathways.

The inhibition of succinate -driven ATP synthesis in rat liver here reported, again raises the question of the lack of sensitivity of normal cells to the drug [3]. However, it must be noted that cancer cells already utilize a truncated version of the Krebs cells, but in a different point of the cycle ; a a truncation point in the tumor Krebs cycle is located between citrate (which is preferentially extruded) and α-chetoglutarate [26-28], and this could make the difference in drug toxicity, with respect to normal cells.

Morover, a major substrate for the energy metabolism of rapidly growing tumour cells is glutamine, which feeds the Krebs cycle following conversion to α- chetoglutarate. Thus, inhibition of succinate dehydrogenase could impair complete utilization of this substrate in tumour cells (glutaminolysis).

Finally, the inhibition of vacuolar ATP ase by 3-BrPA [13] probably plays an important role in 3-BrPA cytotoxicity, considering that the inhibition of this enzyme leads to apoptotic cell death [29]. Thus, the cell death observed following to prolonged (10-15 hours) incubation with the drug [1] could be ascribed to H^+vacuolar ATPase inhibition too.

Perhaps, to explain the reported therapeutic effectiveness of the drug against rat hepatoma, a more attractive suggestion might be that the drug penetrates the cells through the pyruvate-lactate transporter and that 3-BrPA uptake is enhanced in tumor cells due to upregulation of the transporter as occurs for glucose. Evidence of upregulation of the monocarboxylate transporter has been found for neuroblastoma cells [30].

ABBREVIATIONS USED

3-BrPA = 3-Bromopyruvate

Ap_5A = Diadenosine 5 pentaphosphate

AO = Acridine orange

SMP = Submitochondrial particles

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