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Fondazione Policlinico Universitario A. Gemelli
Università Cattolica del Sacro Cuore

Mitochondrial respiratory complexes as targets of drugs

- Critical importance of protocols: intact vs permeabilised cells -

Roberto Scatena

Institute of Biochemistry and Clinical Biochemistry

Catholic University - Rome -



EBEC 2016

European Bioenergetics Conference

Riva del Garda, July 2-7, 2016

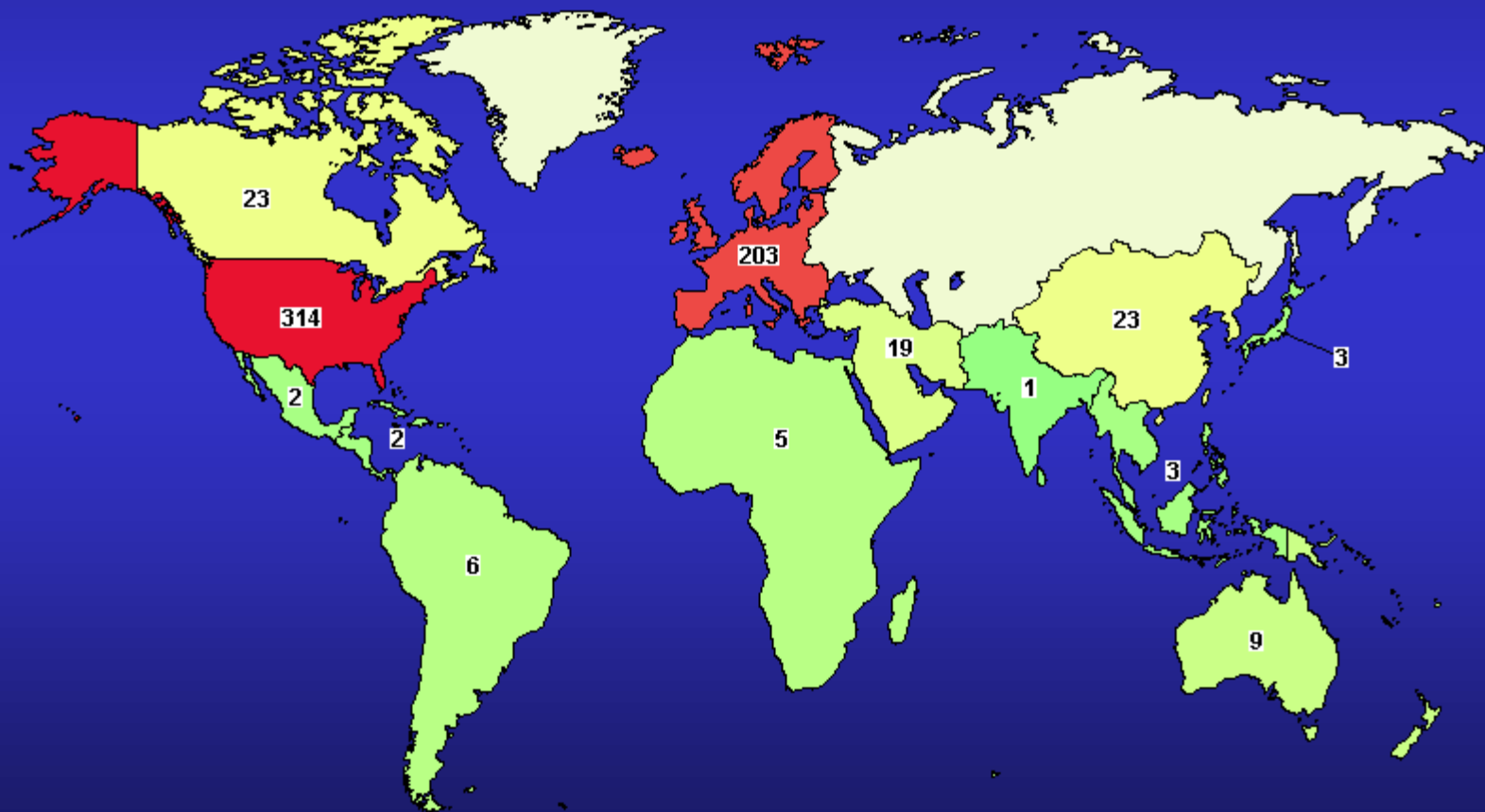


ClinicalTrials.gov

A service of the U.S. National Institutes of Health

**643 studies found
for: mitochondria**

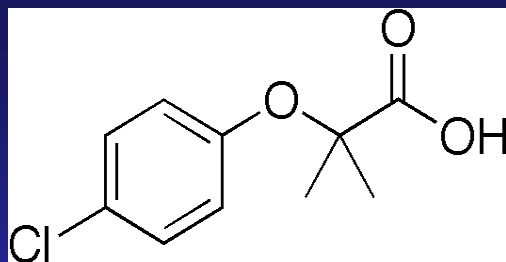
ClinicalTrials.gov is a registry and results database of publicly and privately supported clinical studies of human participants conducted around the world.



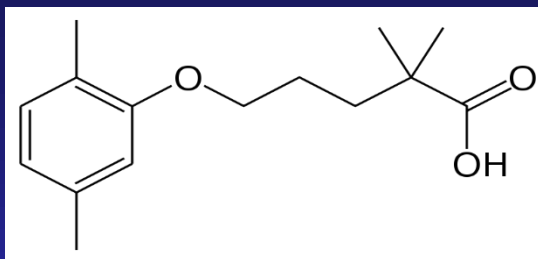
Nov 8, 2016

Mitochondrial respiratory complexes as targets of drugs

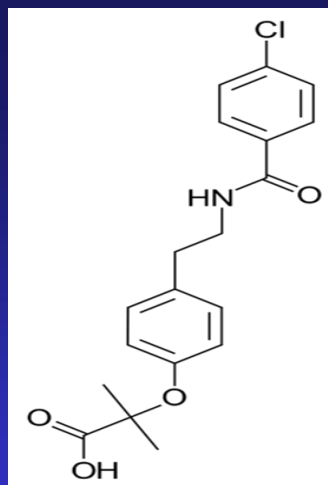
The example of fibrates and glitazones



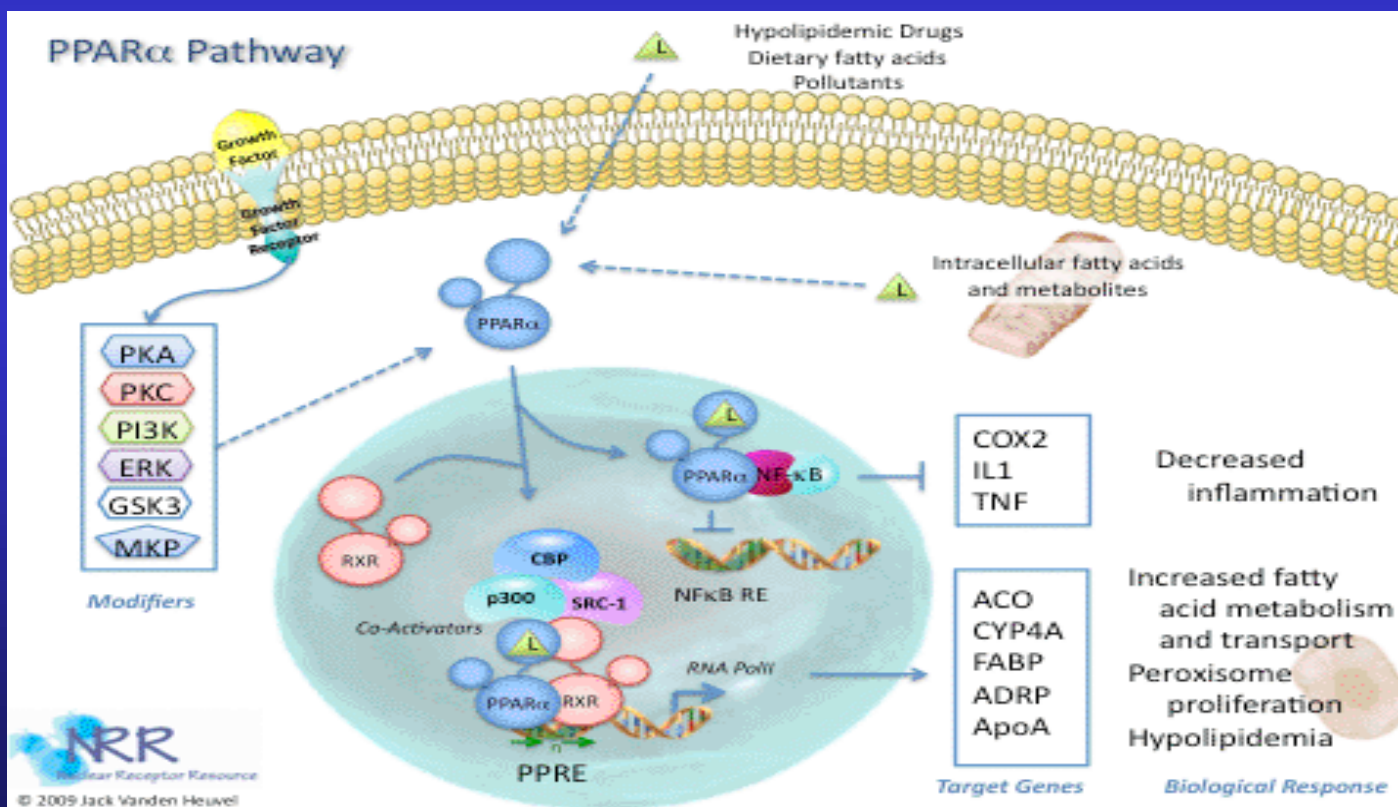
Clofibric acid

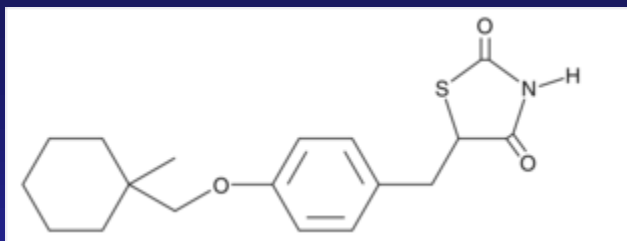


Gemfibrozil

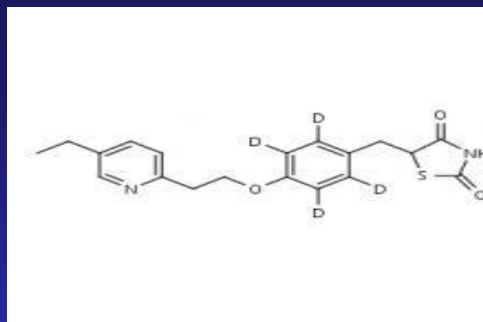


Bezafibrate

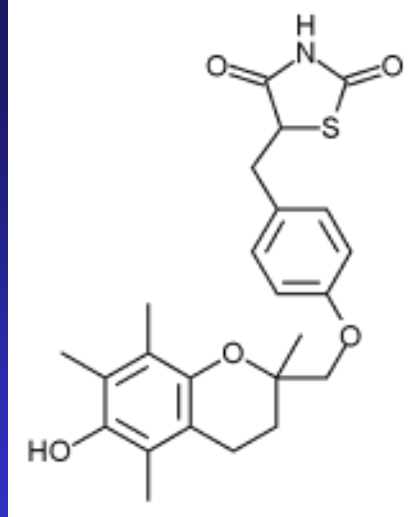




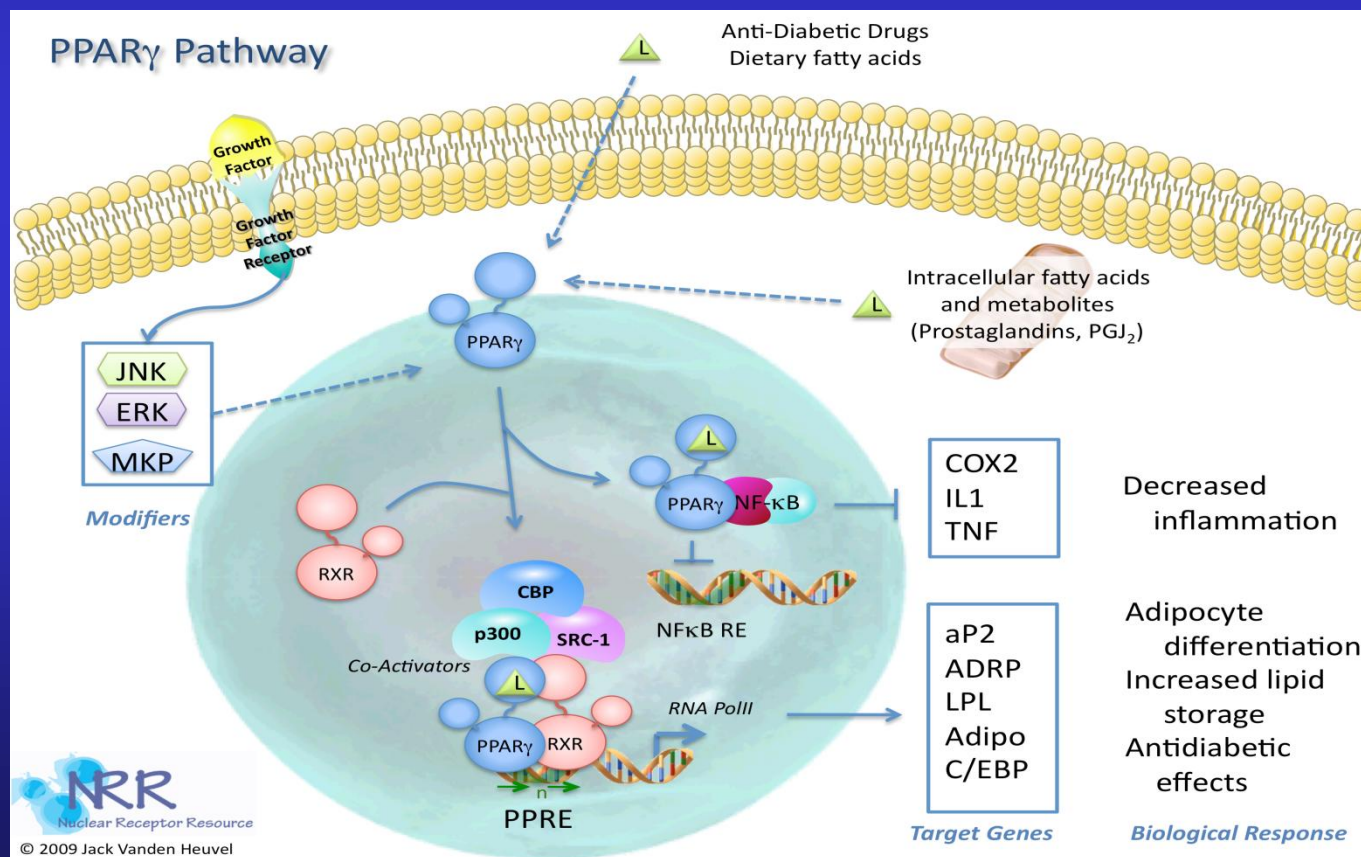
Ciglitazone



Pioglitazone



Troglitazone



Other intriguing (less known and/or considered) biochemical properties such as:

- * Peroxisomes proliferator activity, particularly at hepatocyte level;
- * Hepatocarcinogenic effect in rodents;
- * Allosteric interaction with human hemoglobin with a consequent reduction of oxygen affinity of the hemoprotein;
- * Direct or indirect stimulatory action at the level of genes codifying for cytochrome P450 IV family;
- * Myotonic-like activity, which can induce muscular disorders resembling, from the clinical and pathological point of view, the myotonic dystrophy;
- * an enhancement of the oxidative burst of leukocytes in vitro;
- * an auxinic and herbicide-like activity;
- * a debated inhibitory activity of mitochondrial energetics in vitro and in vivo

Moreover, the following points have to be taken in consideration:

- * Synthetic PPARs ligands are a very heterogeneous class of amphipatic chemicals which display very similar effects.
- * PPAR- α and - γ induce the expression of different gene families related to lipid metabolism (catabolism or storage of fatty acid, respectively).
- * Despite their different physiological roles, synthetic ligands for PPAR- α and - γ actually cause similar therapeutic effects in vivo (hypolipidemic and hypoglycemic effects).
- * A great number of studies demonstrated that synthetic ligands for PPAR- α and - γ display both a differentiating activity and a carcinogenic role on experimental models
- * Paradoxically, these activities, much alike as their metabolic effects are regarded, do not strictly depend on cell or tissue PPAR isotype expression.

Perutz MF, Poyart C. Bezafibrate lowers oxygen affinity of haemoglobin. Lancet. 1983.2, 881-2.

THE LANCET, OCTOBER 15, 1983

Preliminary Communication

BEZAFIBRATE LOWERS OXYGEN AFFINITY OF HAEMOGLOBIN

M. F. PERUTZ C. POYART
MRC Laboratory of Molecular Biology, Cambridge; and INSERM, 92150 Suresnes, France

In a search for possible drugs against sickle cell anaemia, Abraham et al¹ found that clofibrate acid has antisickling properties and shifts the oxygen equilibrium curve of normal human haemoglobin (Hb) to the right.¹ This led us to examine another antihyperlipoproteinaemia drug, bezafibrate ('Bezalip'). Its effect on the solubility of sickle cell Hb is opposite to that of clofibrate acid, so that it would enhance sickling, but it shifts the oxygen equilibrium in the same direction. In fact it lowers the oxygen affinity of normal human Hb more strongly than does the natural allosteric effector 2,3-diphosphoglycerate (2,3-DPG), and it combines with different binding sites in the Hb molecule. Unlike organic phosphates that lower the oxygen affinity, bezafibrate passes freely into and out of the red blood cell, and it does not appear to be bound by serum albumin. These properties may have clinical applications.

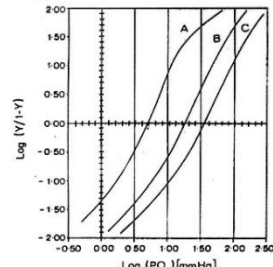


Fig 1—Hill plots of oxygen equilibrium curves recorded with solutions of normal human HbA₁ (as described by Phillips et al²). (A) control without drug or organic phosphates; (B) plus 5 mmol/l bezafibrate; (C) plus 5 mmol/l bezafibrate and 20 mmol/l 2,3-DPG. pH 7.2, 100 mmol/l chloride, 50 mmol/l bis-tris, 25°C. Y = fractional saturation with oxygen.

EFFECTS OF ANTILIPOPROTEINAEIA DRUGS ON OXYGEN AFFINITY OF HUMAN HbA₁ IN ABSENCE OR PRESENCE OF 2,3-DPG

Conditions (mmol/l)	P ₅₀ (mm Hg)	n ₅₀ *	P ₅₀ (HbA-X) (mm Hg)†
Control	5	2.83	-
+ bezafibrate (5)	15	2.61	+14
+ 2,3-DPG (10)	16	2.92	+9
+ bezafibrate (5) and 2,3-DPG (10)	38	2.50	+33
+ clofibrate acid (5)	7.0	2.60	+2
+ 2,3-DPG (10) and clofibrate acid (5)	18.0	2.80	+13

* Hill's coefficient at 50% oxygen saturation.
† (HbA-X) is the difference between P₅₀ in the presence of drug (with or without 2,3-DPG) and P₅₀ for the control experiment.
Bezafibrate = 2-[4-(2-p-chlorobenzoamidoethyl) phenoxy]-2-methylpropionic acid.
Clofibrate acid = 2-[4-(2-chlorophenoxy)-3-methylpropionates].

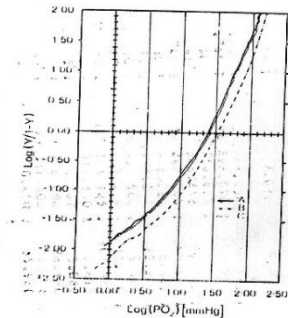


Fig 2—Hill plot of oxygen equilibrium curves recorded with suspension of fresh human red cells from normal donor. (A) control without drug; (B) after 40 min incubation at 37°C in an isotonic buffer containing 5 mmol/l bezafibrate; (C) cells incubated with bezafibrate at 37°C, after washing and incubation with drug-free isotonic buffer at 37°C for 30 min. pH 7.40, 140 mmol/l sodium chloride, 10 mmol/l glucose, 37°C.

Issemann I, Green S *. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature. 347, 645, 1990

We have cloned a member of the steroid hormone receptor superfamily of ligand-activated transcription factors. The receptor homologue is activated by a diverse class of rodent hepatocarcinogens that causes proliferation of peroxisomes. Identification of a peroxisome proliferator-activated receptor should help elucidate the mechanism of the hypolipidaemic effect of these hepatocarcinogens and aid evaluation of their potential carcinogenic risk to man.

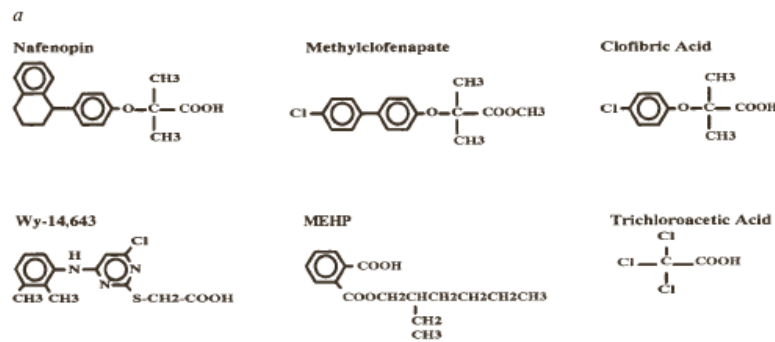
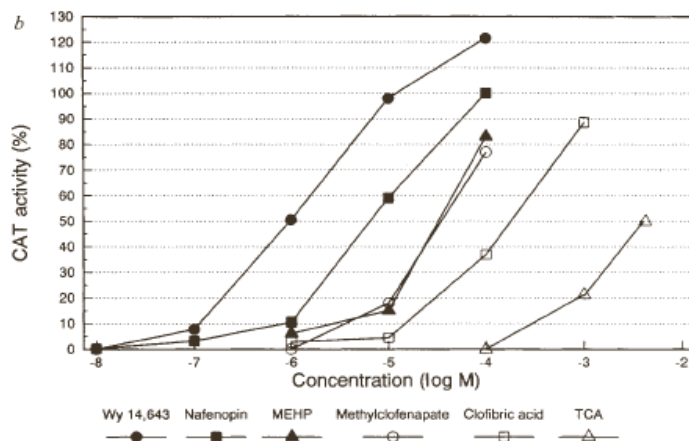


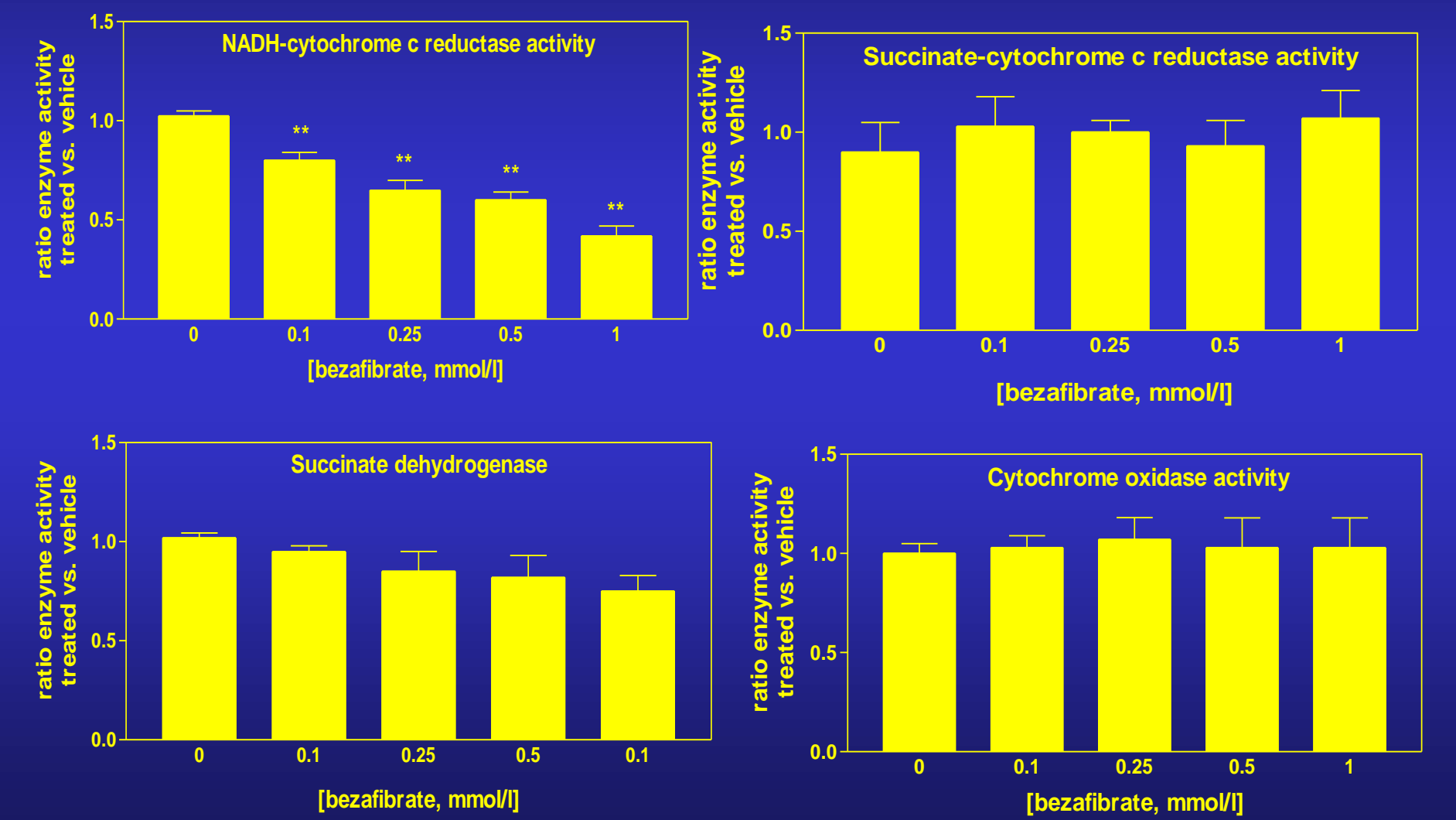
FIG. 6 Dose-response of CAT stimulation using peroxisome proliferators. **a**, Structure of the peroxisome proliferators tested. **b**, Stimulation of Vit-G-CAT by ER-PPAR in presence of peroxisome proliferators. COS1 cell transfections and CAT assays were performed as described in Fig. 5. Spots of acetylated ^{14}C -chloramphenicol were cut out from the TLC plate and quantified by liquid scintillation counting. Each point was performed in triplicate and the values vary by less than $\pm 10\%$. The results are normalized by taking the induction observed with $100\ \mu\text{M}$ nafenopin (typically 10–20-fold) as 100%.



The physiological role and putative natural ligand of PPAR are unknown. The induction of the peroxisomal fatty-acid β -oxidation system by peroxisome proliferators may indicate a role for the receptor in providing a source of energy via fatty acids. Additional possibilities include the regulation of peroxisomal function or cholesterol metabolism, for example by increasing the conversion of cholesterol into bile acids; a step of which is performed mainly, if not exclusively, in the peroxisomes³³.

*Imperial Chemical Industries PLC, Central Toxicology Laboratory, Alderley Park, Macclesfield, UK.

Scatena R, et al . Bezafibrate induces a mitochondrial derangement in human cell lines: a PPAR-independent mechanism for a peroxisome proliferator. *Chem Res Toxicol*. 2003, 16, 1440-7



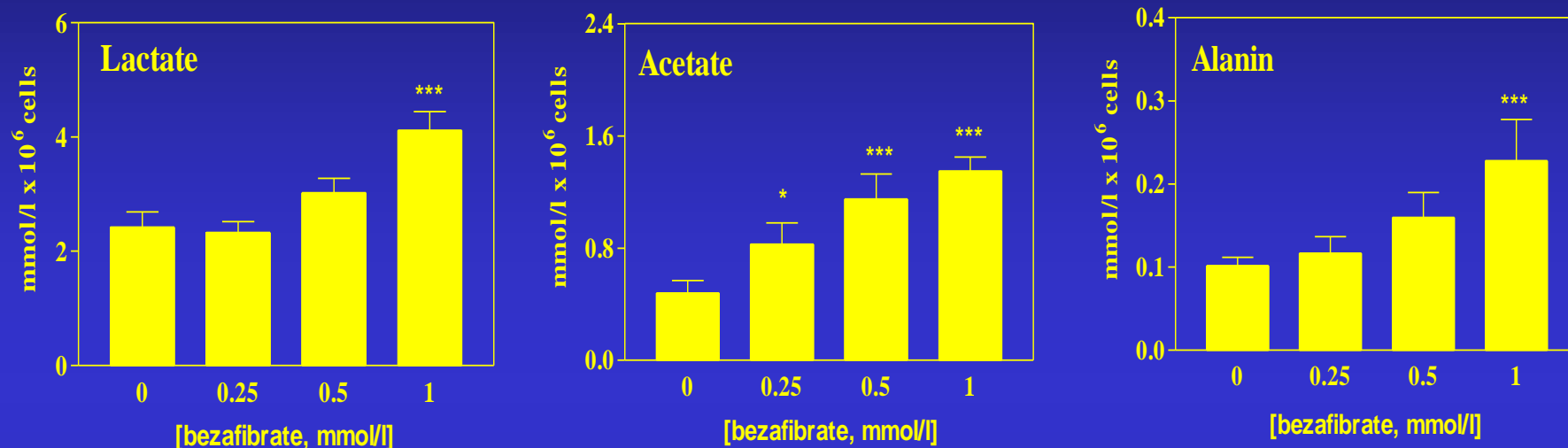


Figure 2. NMR metabolite determinations in supernatant of HL-60 cell cultured for 96 hours in presence of bezafibrate. A dose dependent increase of lactate, acetate and alanin pointed out a compensatory shift to anaerobic metabolism. Results are expressed as the mean \pm SEM, calculated from four experiments, each performed in duplicate. The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls. $p < 0.05$ was considered significant.

*** = $p < 0.001$, * = $p < 0.05$.

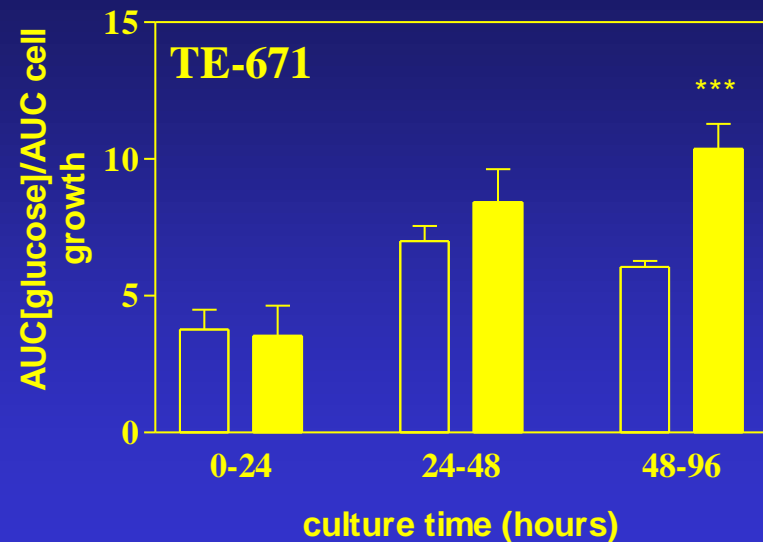
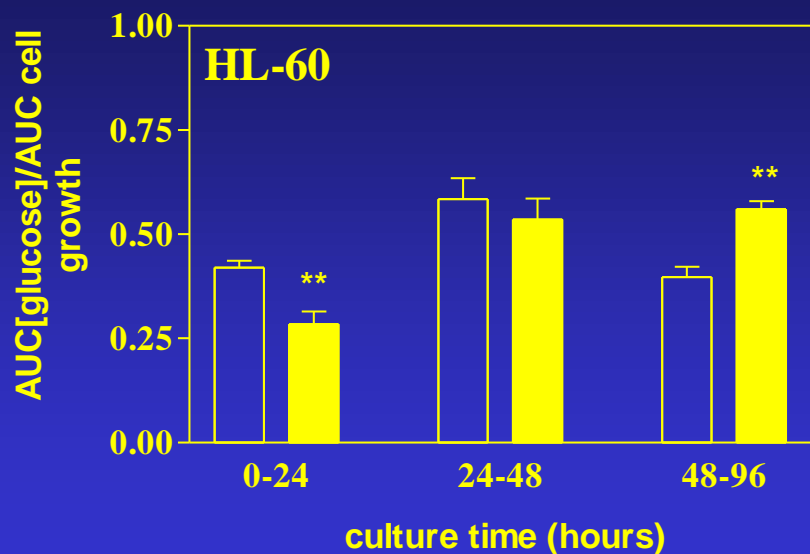


Figure 6. Cell cultured in presence of bezafibrate consumed more glucose than controls though different cell growth kinetics. HL-60 and Te-671 cultured in presence of bezafibrate, 1 mmol/l and 0.5 mmol/l respectively, showed to significantly increase glucose utilization with respect to basal levels consumed. Results are expressed as the mean \pm SEM, calculated from four experiments, each performed in duplicate. The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls. $p < 0.05$ was considered significant. *** = $p < 0.001$, ** = $p < 0.01$.

Thiazolidinediones (TZDs or glitazones): Pioglitazone and Rosiglitazone

► Mechanism of Action

- Enhance insulin sensitivity in muscle, adipose tissue
- Inhibit hepatic gluconeogenesis
- Perhaps, stabilize beta cell dysfunction

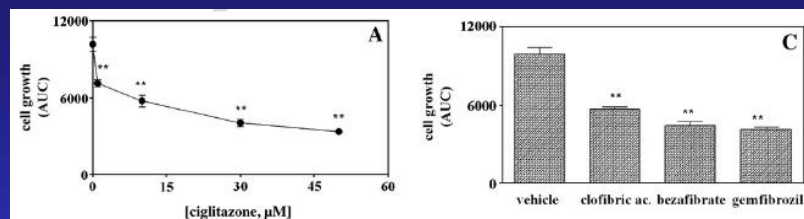
► Safety and Efficacy

- Decrease A1C 1–2%
- **Adverse events:** edema, wt. gain, anemia; worry: liver/heart failure

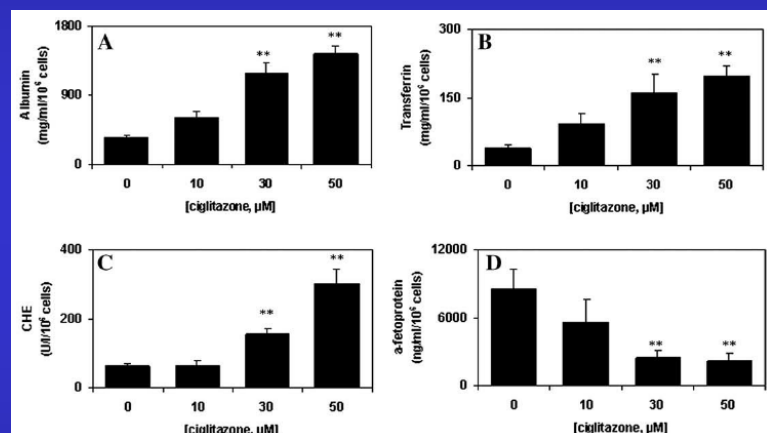
► Dosing

- **Initial dose:** Pio 15 mg qd, Rosi 2–4 mg qd (rosi can be dosed bid)
- **Maximum effective dose:** maximum dose (pio 45 mg qd, rosi 8 mg qd)
- **Titration frequency:** weeks to month(s)

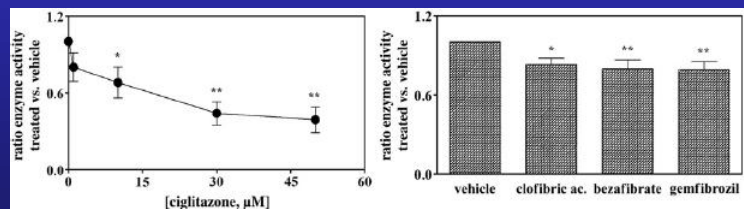
HepG2 cells



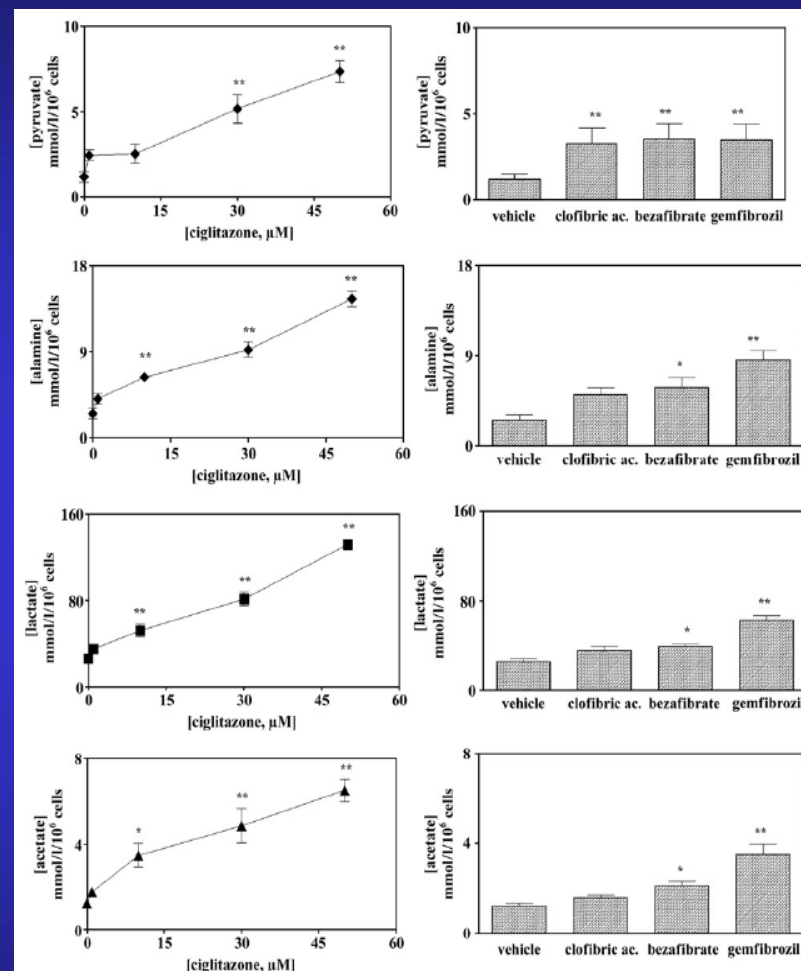
Antiproliferative effect



Differentiating effect



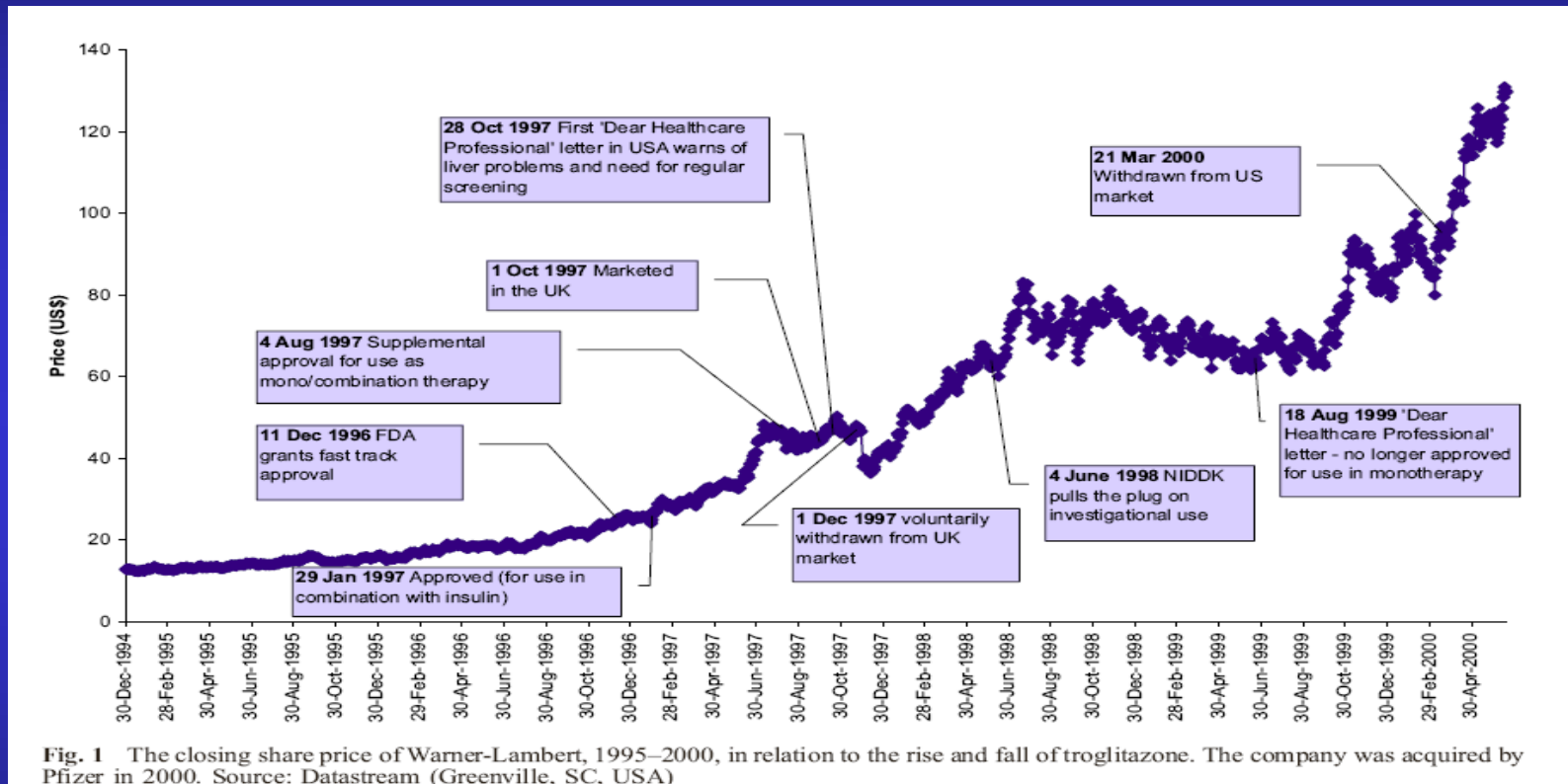
Complex I inhibition



Metabolic alterations

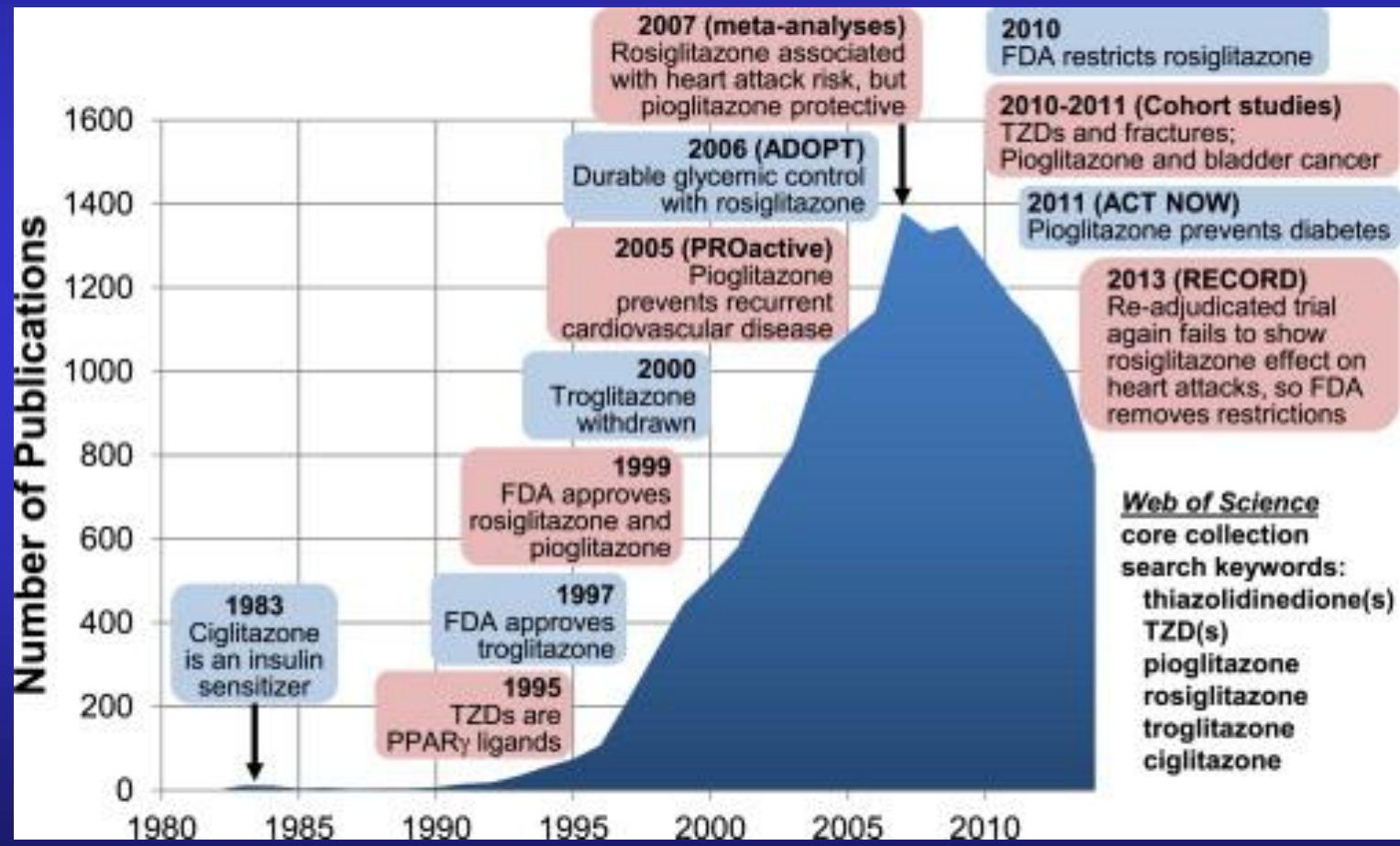
Gale EA. Lessons from the glitazones: a story of drug development. *Lancet*. 357,1870, 2001. Meanwhile it went on to generate sales of over \$2 billion in the USA, and caused at least 90 cases of liver failure (70 resulting in death or transplantation) before it was withdrawn in March, 2000.

Gale EA. Troglitazone: the lesson that nobody learned? *Diabetologia*. 2006 Jan;49(1):1-6.



No inquiry has been held, no one has apologised, no one's career has suffered (with the exception of Richard Eastman), and a lot of people are wealthier in consequence. Clinicians and researchers working with diabetes did well out of troglitazone, and some still talk with nostalgic regret of its wasted potential. The most striking thing about the story is that the medical community remained resolutely silent on the subject of patient safety. No prominent physician, anywhere in the world, ever stood up to say that a pill for diabetes is not worth dying for.

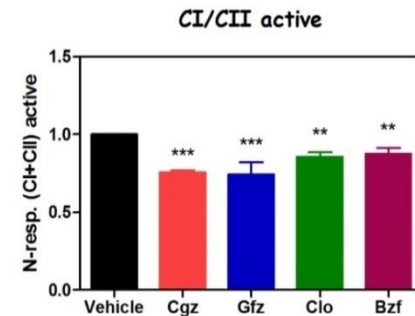
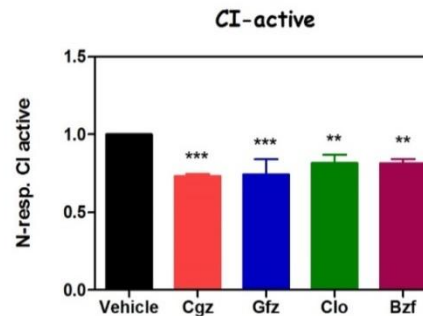
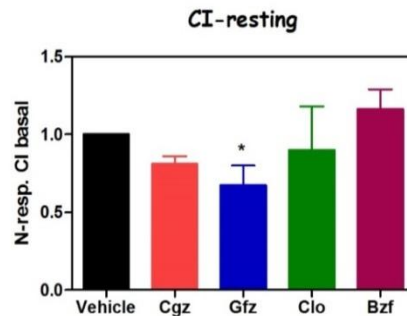
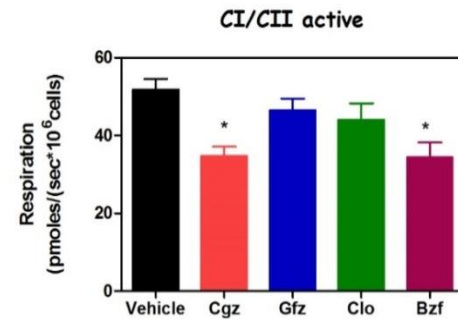
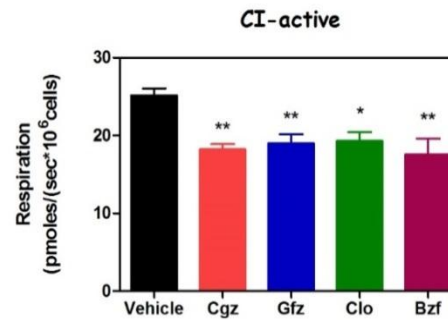
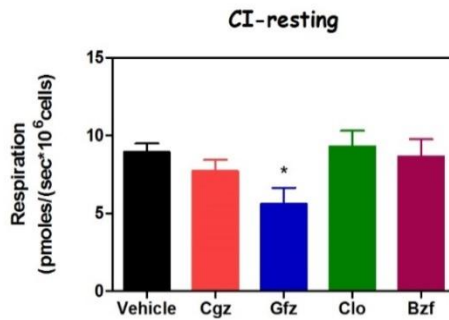
Gale EA. Pioglitazone: are rumours of its death exaggerated?
Diabet Med. 2015, 32, 431-7.





HepG2 cells

Permeabilized cells



CI resting: basal complex I respiration (glut/Mal +Digit)

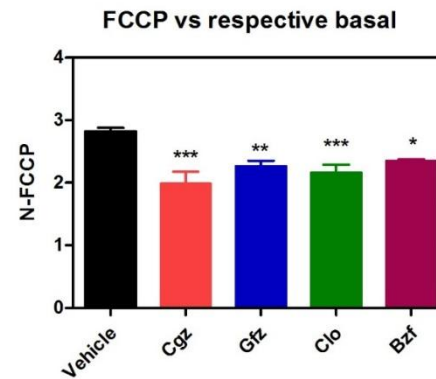
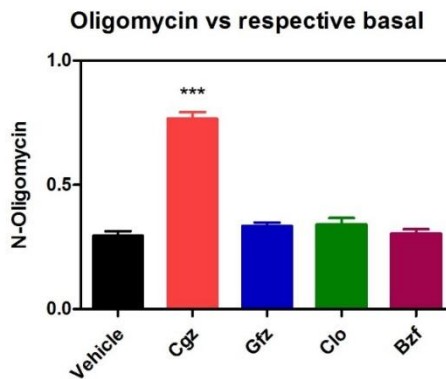
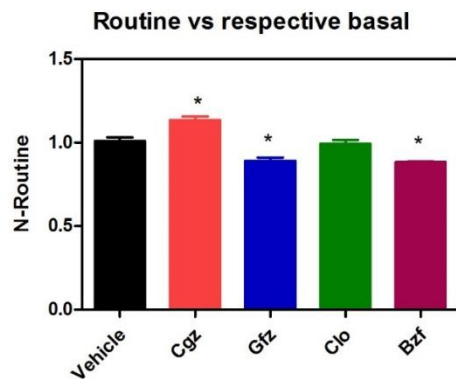
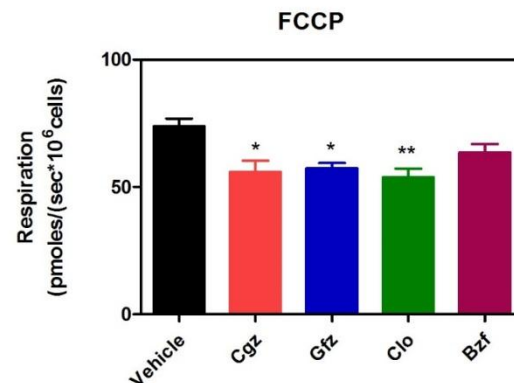
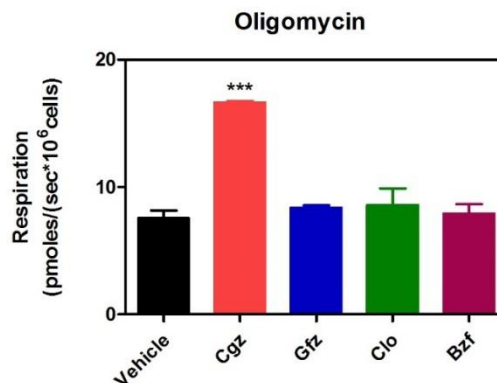
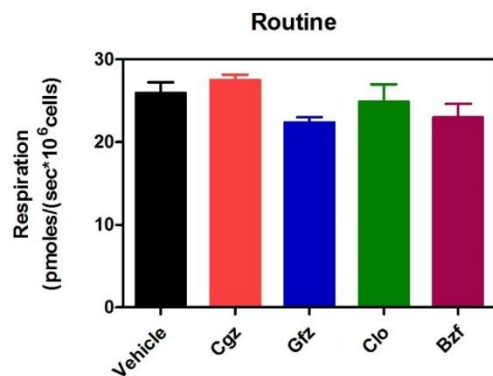
CI active: CI respiration activated by excess of ADP (Glut/Mal +Digit + ADP)

CI/CII active: combined CI and CII respiration (glut/Mal +Digit +ADP+ Succ)



HepG2 cells

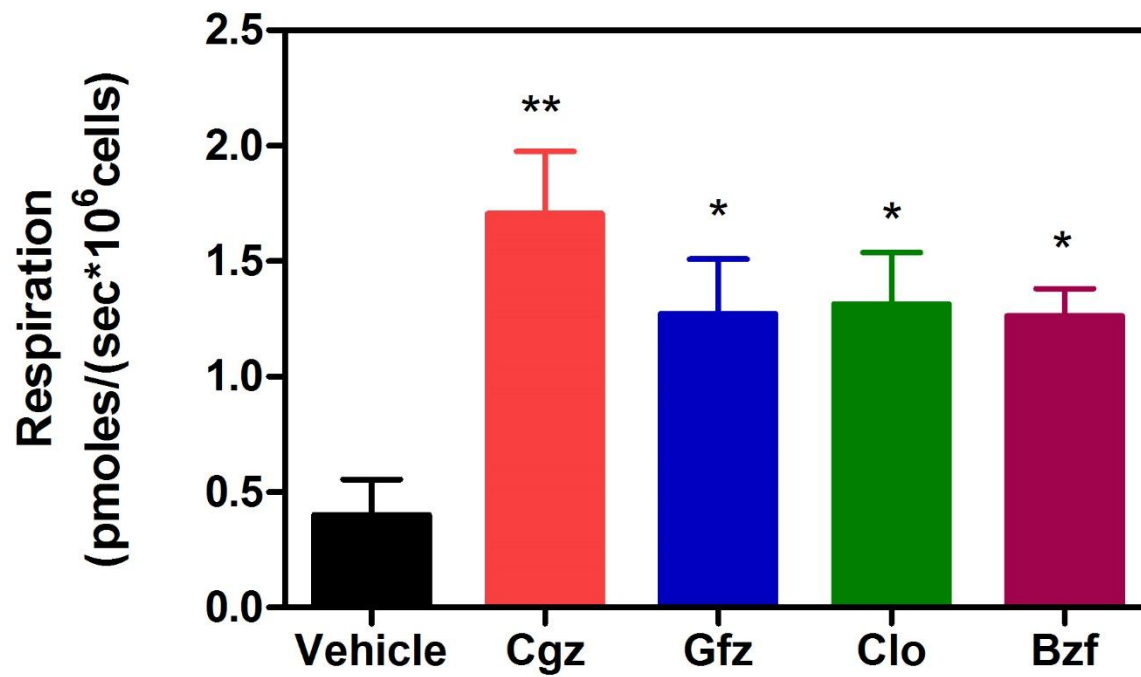
Intact cells



HepG2 cells



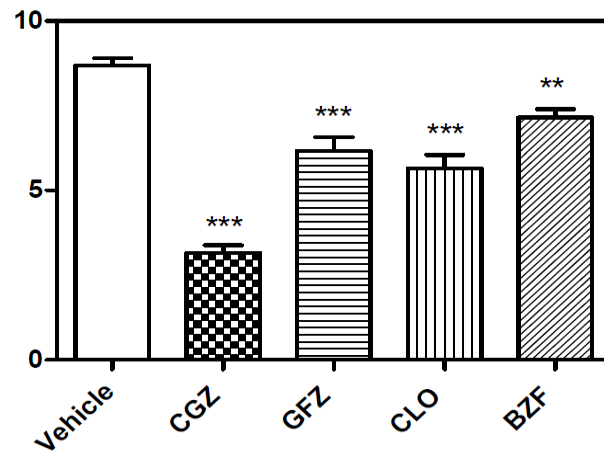
ROX intact cells



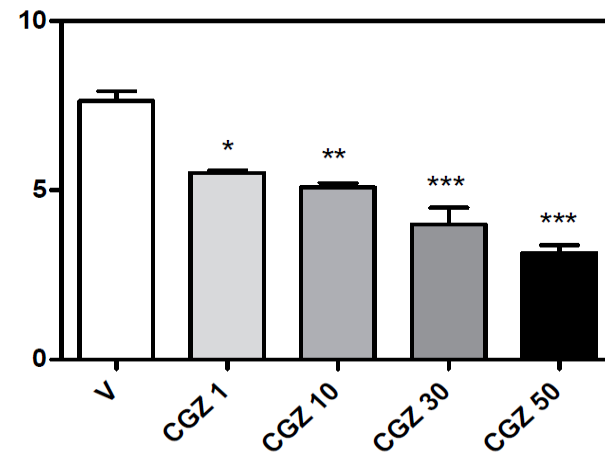
HepG2 cells

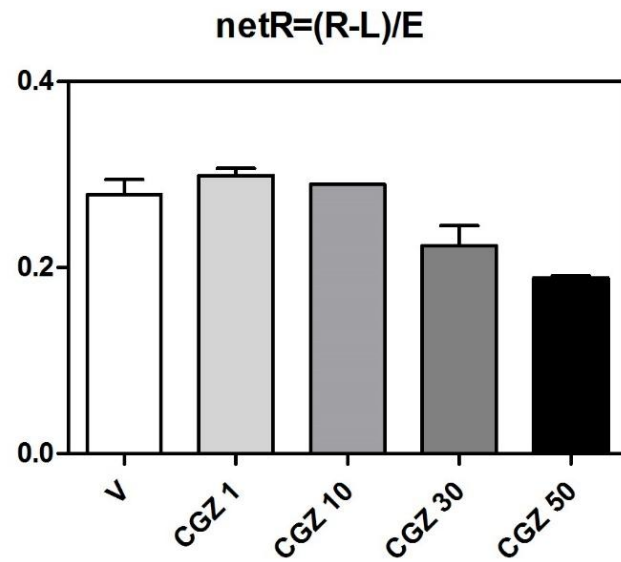
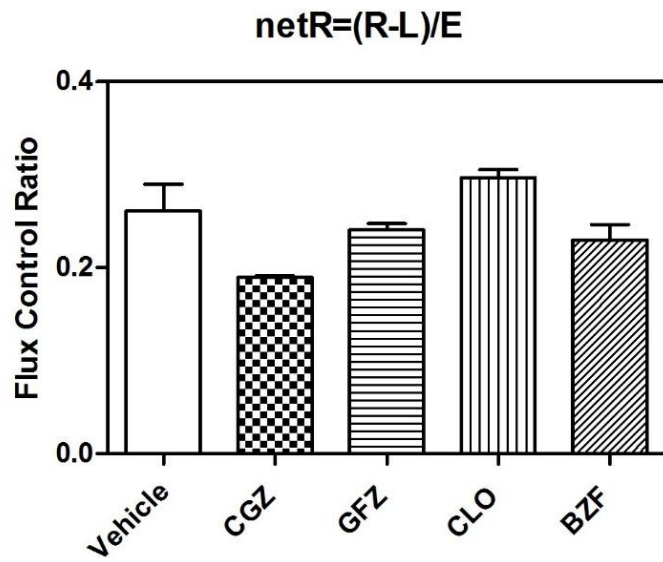
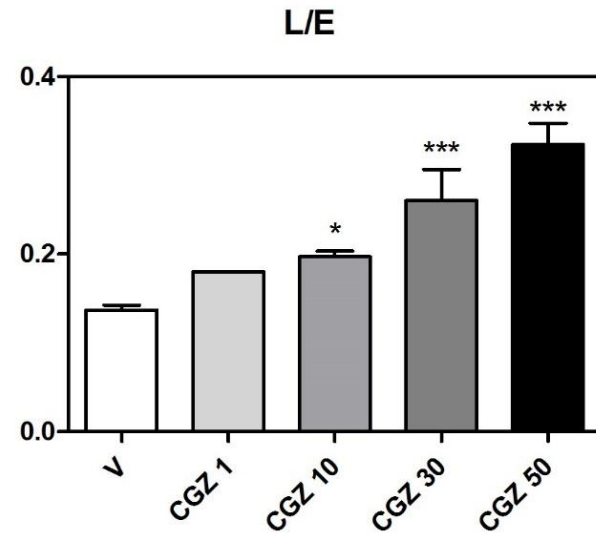
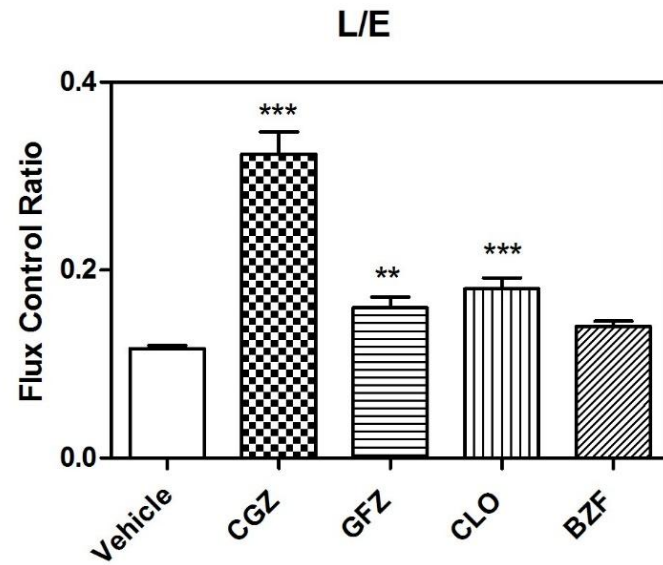


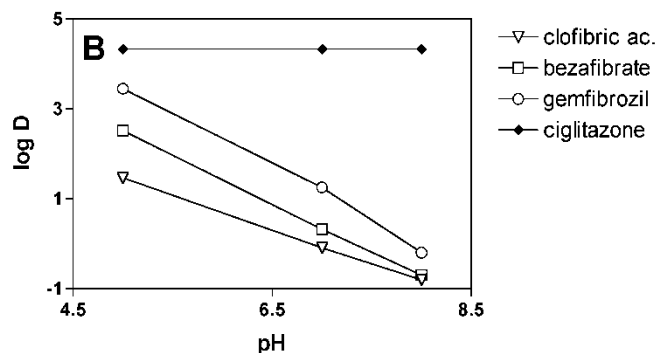
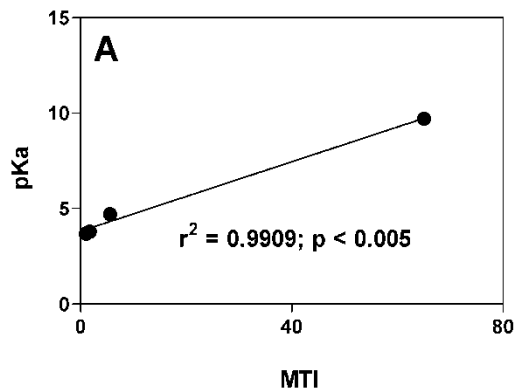
RCR



RCR

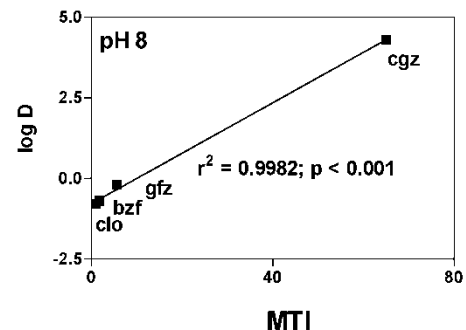
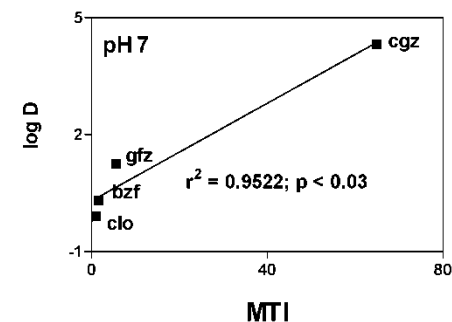
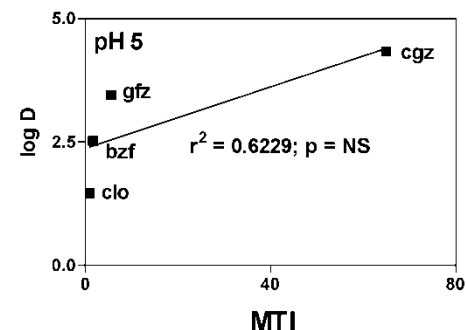






It could be useful to stress that the outlier data (i.e., ciglitazone) seem to give the statistical significance to the whole correlation, while even eliminating data relative to ciglitazone, a statistically significant linear regression persists between mitochondrial derangement and pK values ($r^2 = 0.9981$, $P < 0.03$) and between mitochondrial derangement and log D at pH 8.0 ($r^2 = 0.9998$, $P < 0.01$) of the three remaining drugs. In conclusion, the outlier data only reinforce the statistical significance of the correlation that is already present with only the other three drugs, fibrates.

Figure 5



Clinical implications

The observed mitochondrial derangement should be considered with respect to dangerous side effects such as:

- Rhabdomyolysis (fibrates alone or in association with HMGCoA inhibitors -cerivastatin-)
- Heart failure (thiazodilinediones, i.e. troglitazone)
- Acute liver insufficiency (thiazodilinediones, i.e. troglitazone)
- Kidney : water retention
- Myocardial infarction (rosiglitazone)

•CANCER??

Effets related to prolonged treatments?

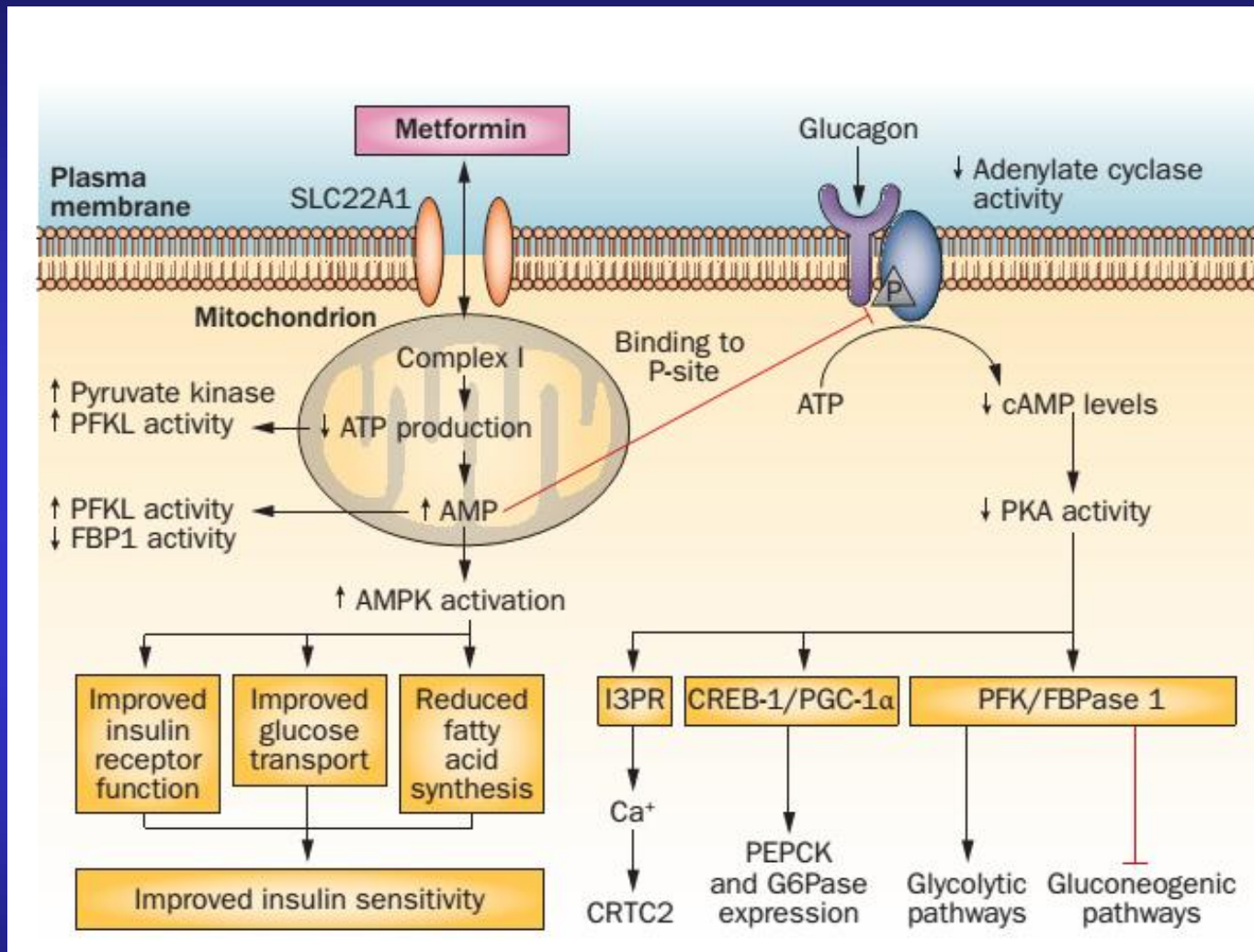
Ingenuity Pathway Analysis (IPA) to assign differentially expressed proteins to functional networks

Tox List	p-value
Mitochondrial Dysfunction	2.74E-04
Renal necrosis/Cell death	1.51E-03
Fatty Acid Metabolism	4.62E-03
Alteration Transmembrane Potential of Mitochondria and Mitochondria membrane	1.27E-02
Oxidative Stress	1.58E-02

Mitochondria dysfunction-associated molecules

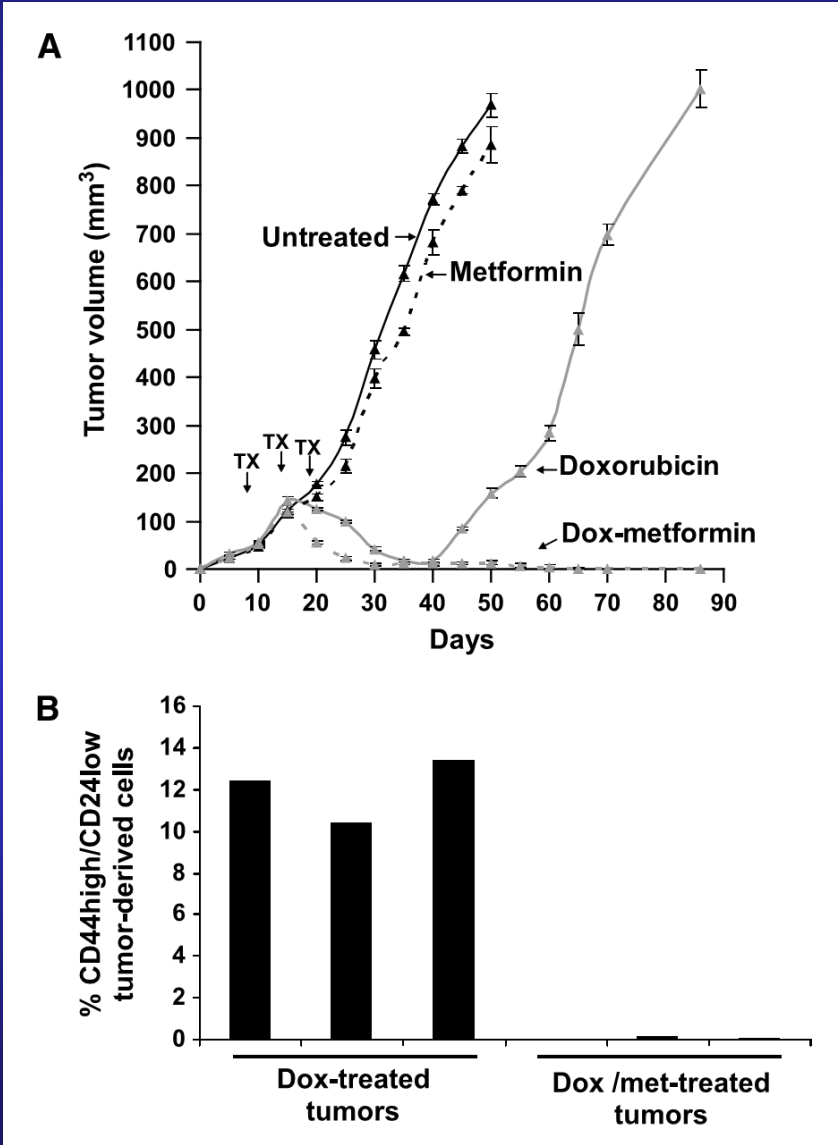
ATP5A1, ATP5O, PRDX3, SOD2, VDAC2

Top Networks	
<i>Associated Network Functions</i>	<i>Score</i>
Neurological Disease, Skeletal and Muscular Disorders, Hereditary Disorder	51
Infectious Diseases, Developmental Disorder, Neurological Disease	48
Cell Morphology, Reproductive System Development and Function, Lipid Metabolism	20
Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	15
Psychological Disorders, Antimicrobial Response, Inflammatory Response	5



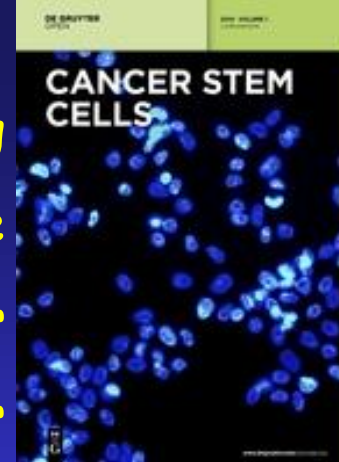
Pernicova I, Korbonits M. Metformin-mode of action and clinical implications for diabetes and cancer. *Nat Rev Endocrinol.* 2014, 10, 143

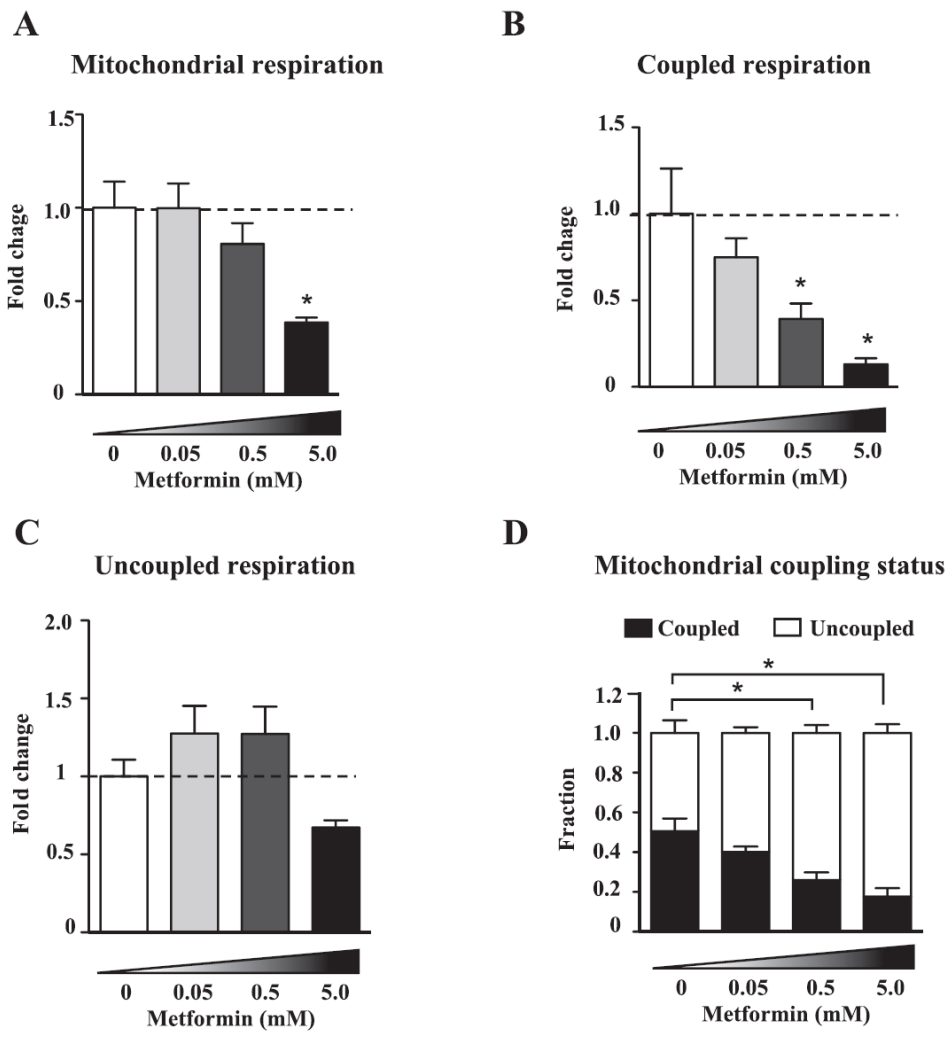
Hirsch HA, et al. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res.* 2009, 69, 7507.



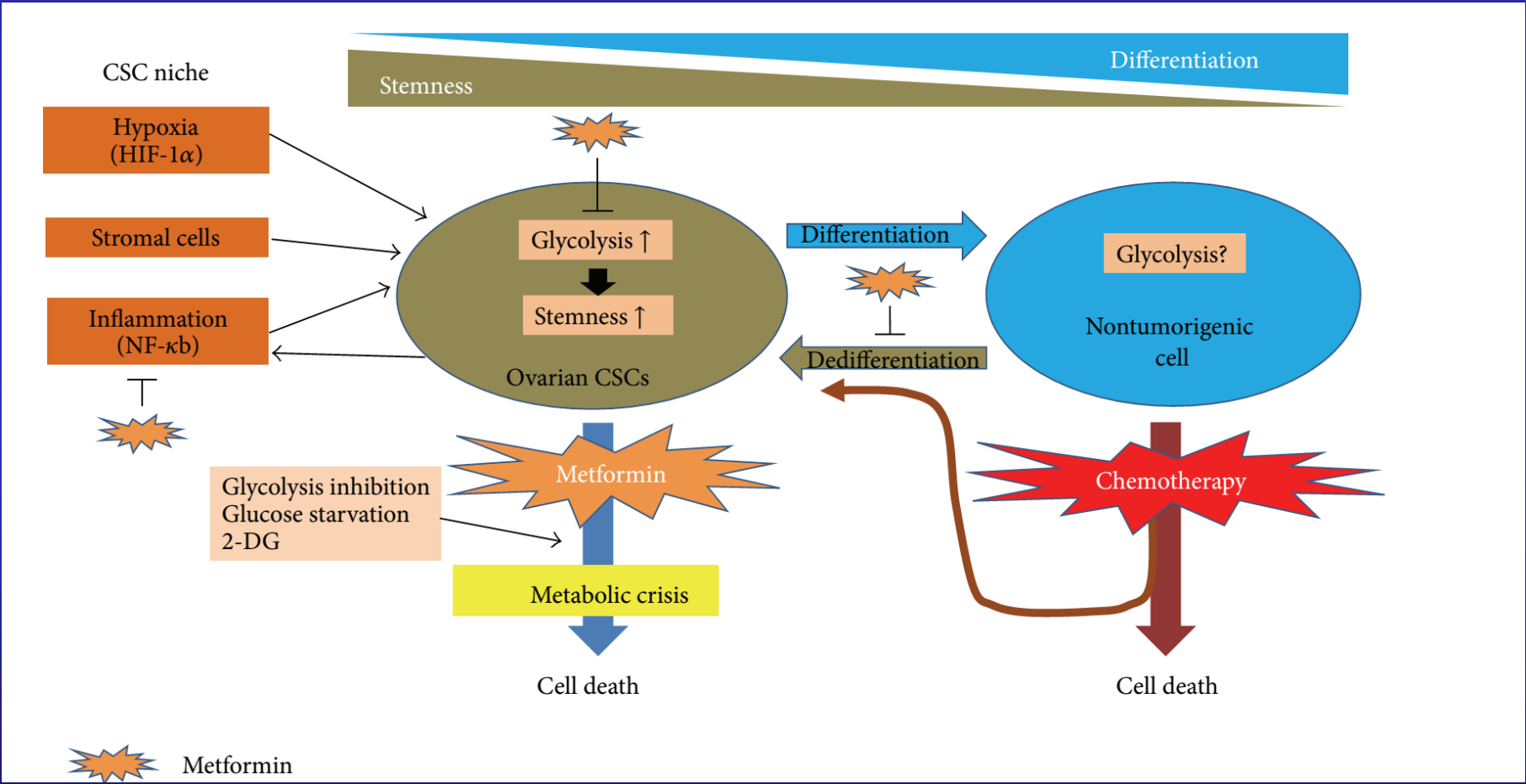
Cancer stem cells (CSCs), or cancer initiating cells (cancer maintaining cells, stem-like cancer cells, pathogenically relevant cancer cells) are distinct from the bulk of the tumor and they are responsible for long-term maintenance of tumor growth

This subpopulation acquired some of the characteristics of stem cells to survive and adapt to ever-changing environments (i.e., ability to self-renew and the capacity to produce progenitors that differentiate in more mature cell types).

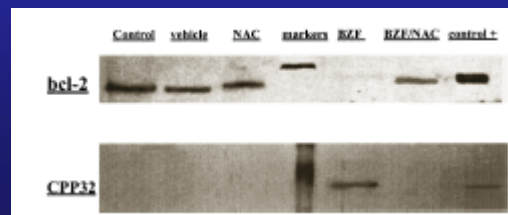
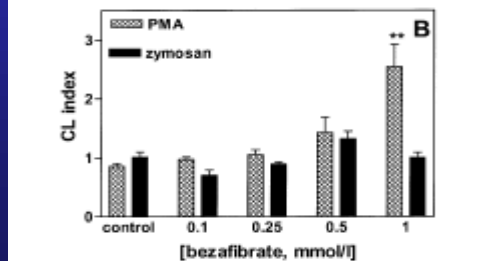
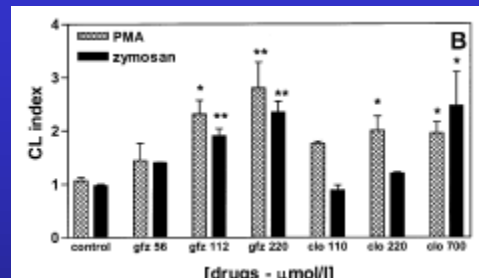
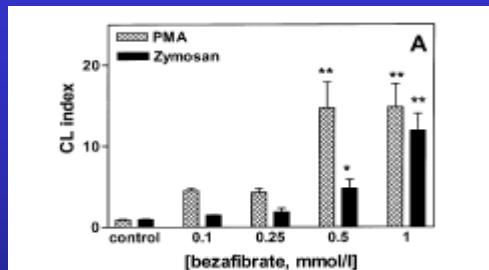
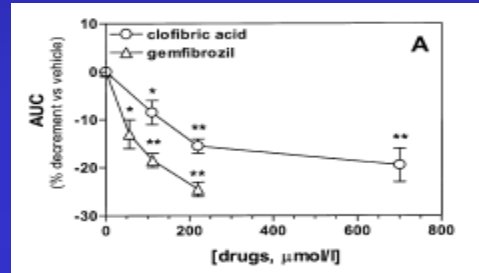
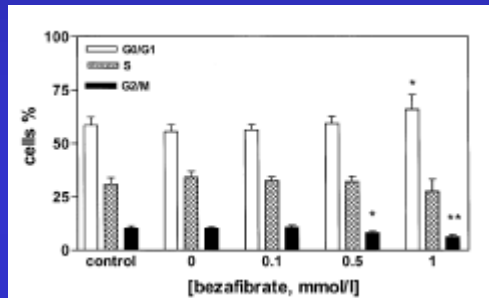
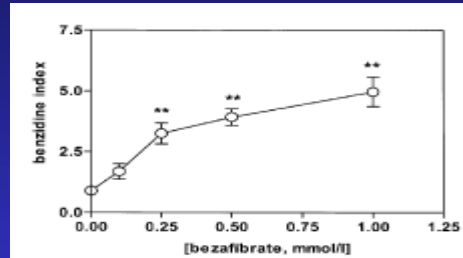
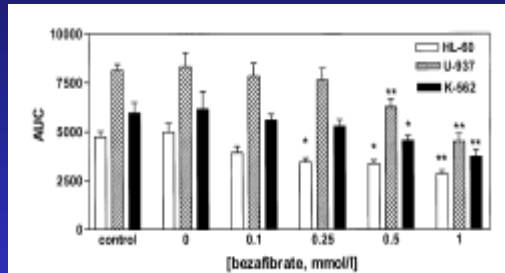




Kim TH, et al. Metformin against cancer stem cells through the modulation of energy metabolism: special considerations on ovarian cancer. Biomed Res Int. 2014;2014:132702.



Scatena R, et al. Bezafibrate as differentiating factor of human myeloid leukemia cells. *Cell Death Differ.* 6, 781, 1999



The precise mechanism of mitochondria damage and its consequence in terms of cellular oxidative metabolism imbalance;

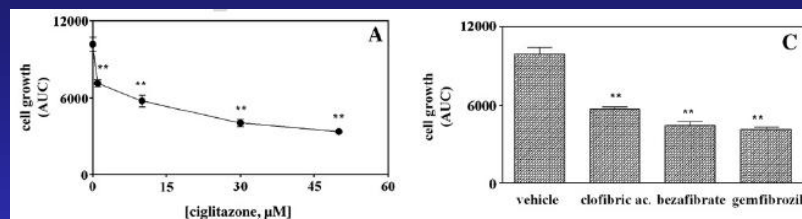
The interrelationships between peroxisome proliferator activity and mitochondrial damage

The compensatory machinery (essentially in terms of HSP induction) implicated in the maintenance of cellular homeostasis and its role in promoting the differentiation state;

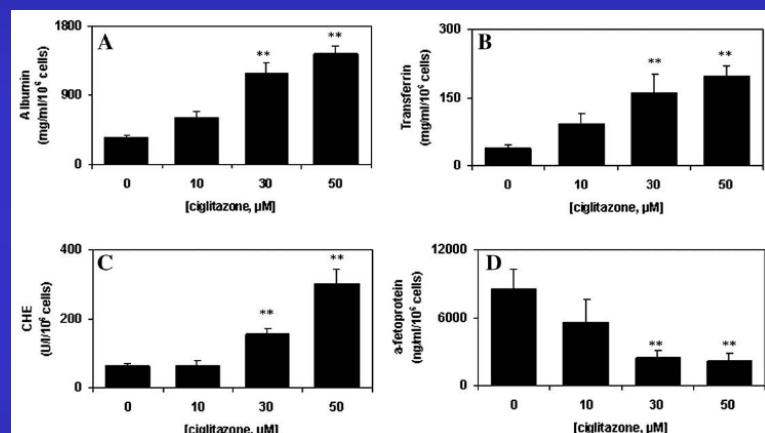
The meaning of such a compensatory mechanism in a peripheral modulation of tumour suppressor genes and/or oncogenes expression;

The role of this particular class of differentiating factors in a new definition of dedifferentiation/differentiation processes in cancer.

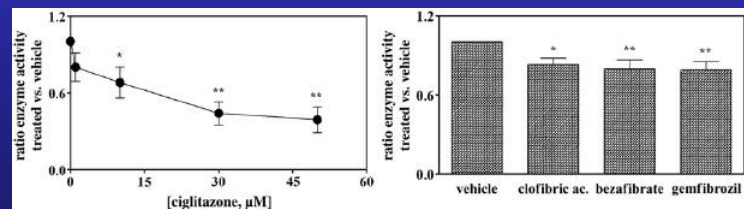
HepG2 cells



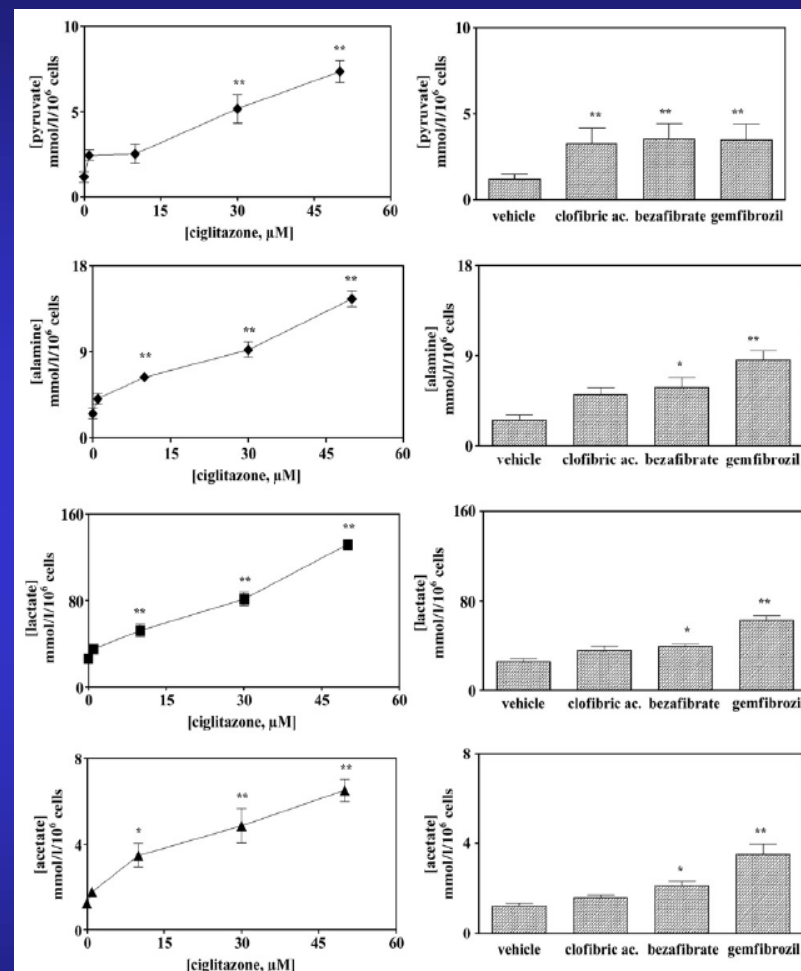
Antiproliferative effect



Differentiating effect



Complex I inhibition



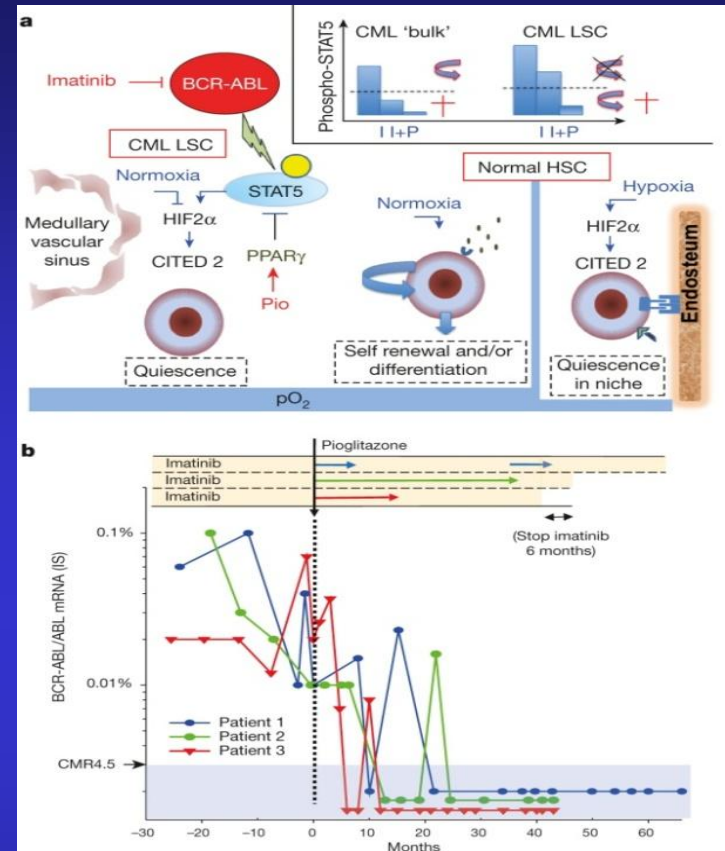
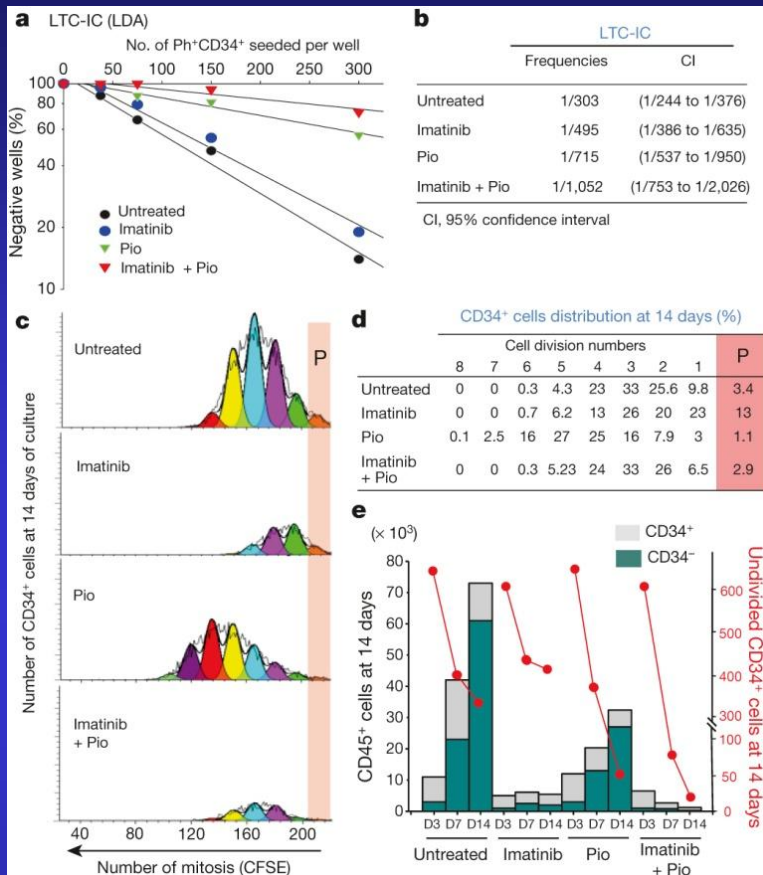
Metabolic alterations

Hep-G2	Mitochondria damage index	Lactate index	AntiProliferative index
Bezafibrate	1.7 ± 0.5	2.8 ± 0.2	0.9 ± 0.1
Clofibric acid	2.9 ± 0.9	4.8 ± 0.8	5.3 ± 0.8
Gemfibrozil	9.5 ± 2.7	12.5 ± 0.6	8.2 ± 0.4
Ciglitizone	$111.2 \pm 17.3^*$	$102 \pm 5.2^{**}$	102 ± 4.1
		$r^2 = 0.9989$ $p = 0.0001$	$r^2 = 0.9991$ $p = 0.0001$

Table II. Quantitative comparison, on molar ratio basis (drug activity/[drug] x 100), between different PPARs ligands and gemfibrozil in terms of inhibition of NADH cytochrome c reductase activity, lactate and antiproliferative index on Hep-G2 cell line. Results are expressed as the mean \pm SEM, calculated from four experiments, each performed in duplicate. The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls. $p < 0.05$ was considered significant. * = $p < 0.05$; ** = $p < 0.01$.

Ciglitizone [μM]	Mitochondria damage index	Lactate	AntiProliferative index
0	0	16.4 ± 0.7	0
10	32.0 ± 0.1*	41.3 ± 0.8 **	46.0 ± 25.9**
33	56.0 ± 1.1**	44.7 ± 0.6**	52.0 ± 24.9**
50	61.0 ± 0.2**	50.9 ± 5.2**	52.0 ± 8.0**
		r² = 0.9266 p = 0.037	r² = 0.7938 p = 0.1091

Table III. Dose dependent effect of ciglitizone in terms of inhibition of NADH cytochrome c reductase activity, lactate production and antiproliferative index on Hep-G2 cell line. Results are expressed as the mean ± SEM, calculated from four experiments, each performed in duplicate. The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls. $p < 0.05$ was considered significant. * = $p < 0.05$; ** = $p < 0.01$.



Pioglitazone purges quiescent CML stem cells

Pioglitazone induces complete and sustained molecular response (CMR) in CML patients

Prost S et al. Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature*. 2015 , 525, 380

But this is a different mitochondrial story...

Thank you

Thiazolidinediones

- Possible side effects:
 - Edema (particularly peripheral edema)
 - Weight gain
 - Headache, Weakness
 - Nausea, vomiting, abdominal pain
 - Bone fracture
 - Increase the risk of heart failure
 - Liver problems (RARE)
 - **Pioglitazone associated with small risk of bladder cancer**
 - Potential myocardial infarctions (rosiglitazone)



